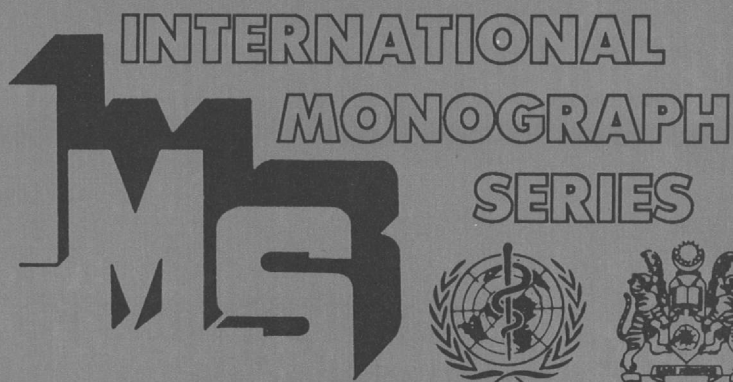


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**The Validation of Chemical and
Immunological Tests for
Antimalarials in Body Fluids:
Papers Presented at a W.H.O./
Universiti Sains Malaysia Workshop**

Pusat Penyelidikan Dadah dan Ubat-Ubatan
(Centre for Drug Research)
W.H.O. Research and Training Centre
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11800 Penang,
MALAYSIA.

**WORLD
HEALTH
ORGANIZATION**

**THE VALIDATION OF CHEMICAL AND IMMUNOLOGICAL TESTS
FOR ANTIMALARIALS IN BODY FLUIDS:
PAPERS PRESENTED AT A WHO/UNIVERSITI SAINS MALAYSIA WORKSHOP**

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INTRODUCTION TO THE PROCEEDINGS OF THE
TDR/CHEMAL WORKSHOP ON THE FIELD VALIDATION OF
CHEMICAL AND IMMUNOLOGICAL TESTS FOR ANTIMALARIAS
IN BODY FLUIDS

16 - 21 October 1989

Following the recommendations of the Workshop on Clinical Pharmacology of Antimalarial Drugs, which was held in Penang from 23 January to 4 February, 1989, the Steering Committee of CHEMAL, UNDP/World Bank/WHO Special Programme of Research and Training in Tropical Diseases (TDR), authorized the organisation of a follow-up Workshop on Chemical and Immunological Tests for Antimalarials in Body Fluids. The objectives of the workshop were to carry out, under strictly controlled laboratory conditions, a comparative evaluation of the available chemical, chromatographic and immunological tests to determine their comparative efficacy in the detection of chloroquine and mefloquine in body fluids, and their applicability to field research and malaria control operations.

The venue was once again located at the National Drug Research Centre of the Universiti Sains Malaysia, Penang, from 16th to 21st October 1989. The co-ordinator of the course was Dr. V. Navaratnam, Director, National Drug Research Centre (NDRC) and through the auspices of the Vice Chancellor of the University, Datuk Hj. Musa Mohamad, all the necessary work space and resources of material and manpower to run the course was provided at the NDRC. The secretariat of the World Health Organisation was represented by Dr. D. Payne, MAP/RTI and TDR. CHEMAL/TDR provided financial support for travel, per diem and local organisational costs.

Prominent researchers in this field from overseas, and within Malaysia, (see list of participants for details), were invited to participate in the workshop, and the whole range of available chemical, chromatographic and immunological test systems were discussed and evaluated. In particular, selected test systems were comparatively examined in the laboratory using standard biological material supplied by NDRC. These included: chemical (CDC - Saker/Solomons tests for chloroquine in urine, CQ I and CQ II, and Haskins MMII); chromatographic (Thin Layer Chromatography - TLC - tests with urine for chloroquine, quinine and mefloquine in urine) and immunoassays (ELISA) using mono- and polyclonal antibodies against chloroquine, quinine and mefloquine. Standards were provided by High Performance Liquid Chromatography (HPLC) assays as routinely practised by the NDRC.

These experiments produced evidence that field test kits using all three methodologies were feasible, and it was recommended that prototype test kits for 100 tests of each type, covering chloroquine and quinine and, where feasible, mefloquine, be prepared for further evaluation in a field application. Should this evaluation produce one or more practical field tests, it was proposed that a further workshop

would be organised at which representatives of all the malarious regions would be present, and after adequate training, these representatives would be supplied with test kits to take back to their home countries for evaluation. Hopefully, this would then lead to the production and distribution of standardised field test kits for chloroquine, quinine and mefloquine in urine and/or blood with the potential for the development of further tests for other current and candidate antimalarial drugs.

THE UTILITY AND POSSIBLE IMPLEMENTATION OF FIELD TESTS FOR THE DETECTION OF AVAILABLE AND NEW ANTIMALARIA DRUGS FOR BODY FLUIDS

David Payne

Introduction

The quest for simple, sensitive and specific tests for the detection of antimalarial drugs in body fluids has a long history. This search has still to reach full realization in that a test meeting all three of the above criteria, for even one of the common antimalarials, has yet to be accepted as a standardized basic test for utilization at the field level.

Moreover, the raison d'etre for such a test has changed somewhat in recent years.

The original requirement was for a test to detect the presence of adequate levels of an antimalarial drug, post-treatment, and to eliminate from drug evaluation trials those candidate subjects who had already had a significant intake of the test or other antimalarial drug.

The current situation, where there is a widening use of second- and third-line, antimalarials, having various known adverse reactions in some subjects, and which may be exacerbated by over dosage and/or use in combination with other drugs, means that there is also an increasing need to know the levels of current medication of a patient, vis a vis the parasitological status of the patient, before embarking on any antimalarial treatment. For some drugs, such as quinine or chloroquine, an approximate estimation may suffice, whereas others, such as mefloquine and sulfadoxine, may need more precise quantification. Additionally, it may be prudent to monitor the levels of the drug in the biological fluids of the patient, during the period of treatment, to ensure that these do not achieve unacceptable levels or, if alternative therapy is considered, to ensure that this is not contraindicated by the drug levels of the earlier medication(s).

Utility of Tests for the Detection of Antimalarial Drugs

As mentioned in the Introduction, the utility of the detection tests for antimalarial drugs has considerably widened. This is not only due to the employment of a wider range of the armamentarium of established antimalarials, and the introduction of new drugs, but also through the development of additional techniques for field evaluation of their use.

Currently the range of utility includes:

- selecting suitable subjects with malaria for in vivo/in vitro studies, monitoring and evaluating the results of these tests, and following-up patients in retrospective studies.
- comparing the levels of the drug in the body fluids of a malaria patient and the response, in parasitological terms, of the infection to the drug
- monitoring treatment of a malaria patient to ensure that levels of the drug do not exceed optimum levels and that additional, or alternative, treatment is not contraindicated
- providing information on what levels of drug have been absorbed, when vomiting and/or diarrhoea has occurred, to facilitate decisions on further treatment
- evaluating the utilization of a drug in the community and establishing patterns of consumption.

The collection of test samples at the field level may present considerable logistic problems and is frequently complicated by customs and taboos. To this must be added the increasing, but understandable, reluctance of the general public to accept invasive blood sampling techniques of any kind.

Accordingly, the extent at which a particular test is utilized in a field programme will be much influenced by the ease with which samples can be collected, transported and ultimately processed. A lower degree of precision may be acceptable if a higher degree of acceptance and reliability is obtained.

Since the various common antimalarial drugs differ enormously in the way they are selectively absorbed and excreted from the human body, the detection of a particular test drug requires a clear understanding of the distribution and metabolism of the drug.

The collection procedure to be employed, and the reliability of the test system, will be dependent on the persistence, calculated in terms of the half-life of the drug, in the test sample at the time of collection.

The variability of these half-life thresholds can be seen from the table below which gives the values for nine common antimalarials: (WHO, 1984, 1986; Davidson, 1989).

HALF-LIVES OF COMMON ANTIMALARIALS IN THE PLASMA OF THE BLOOD

chloroquine	60 to 168 hours
dapsone	26 hours
halofantrine	110 hours
mefloquine	360 to 792 hours
primaquine	6 hours
proguanil	24 hours
pyrimethamine	80 to 100 hours
quinine	10 to 12 hours
sulfadoxine	100 to 200 hours

From the above it is obvious that to obtain a minimum threshold of a particular drug in the plasma, which would determine the acceptability of a patient for inclusion into a drug trial, it is not feasible to use a standard time period, say 14 days, since earlier medication with five of these drugs, (chloroquine, halofantrine, mefloquine, pyrimethamine and sulfadoxine), within this time-frame would still provide significant quantities of the drug in the plasma whilst the other four drugs would be at relatively insignificant levels. Accordingly, each drug requires a specific test procedure which matches the known pharmacokinetic characteristics of that drug.

Implementation of Field Tests

Material for the detection of antimalarial drugs in the human body is obtainable from six sources: tissue, faeces, urine, whole blood (providing red blood cells and/or plasma and/or serum), breast milk and hair (chloroquine studies).

Detection of the drug in the faeces is feasible, but depends on the metabolism of the drug, whilst the urine is generally more productive and more convenient to process; the complications of tissue sampling are obviously self-exclusive.

Blood samples require an invasive technique and, where volumes in excess of 200 - 300 microlitres are essential, venepuncture is necessary with all the manifold operational difficulties that may entail. However, unless there is a strong local aversion to all blood sampling techniques, finger stick samples are readily collectable and provide excellent standardizable samples which can be processed on-the-spot or, if technically essential, or operationally desirable, transported in the collection tubes. They can also be dried onto filter paper for elution and analysis at a later date. This date can be, in some test procedures, several months later, which introduces the possibility of centralized testing procedures with the advantages of scale, economy and standardization that this affords.

Overall, urine samples usually provide the simplest routine methods of collecting samples, and are generally well accepted by the general public, provided due attention is paid to cultural sensibilities and appropriate action is taken to ensure that these are seen to be catered for. Very small children are difficult to monitor unless special provision is made for appropriate urine collection devices and the mothers of the children are properly briefed on how they are to be used. However, monitoring of the urine collection process, to ensure that there is no mixing or substitution of the samples, is not always easy, particularly when there are women and children involved. When blood samples are obtained there is usually a better positive correlation, and more consistency, between the samples and the donors.

The need for the involvement and participation of the local community, and particularly its more influential members, in all aspects of the planning for, and the implementation of, field trials for the detection of antimalarials in body fluids is self evident. It is essential that due attention is paid to this important aspect of the work.

Specificity and Sensitivity of Field Tests

Specificity has usually been understood to be, and interpreted as, detection of positivity, degree of reliability and lack of cross-activity with common antimalarial and other drugs.

Sensitivity, on the other hand, has meant resolution and limit, or threshold, of detection.

The ideal test would be, therefore, one which was specific to the drug under test, and no other commonly used drug. It should also have an adequate degree of reliability, whilst being of sufficient sensitivity to ensure that levels of the drug meaningful under the required test conditions would always be detected.

Conclusions

1. The operational practicalities limit a field test for antimalarials to samples of urine or blood from a finger stick.

A technique which could utilize blood samples dried onto filter paper, for later elution and processing, would probably offer more potential than one limited to only fresh blood.

2. Tests which are specific for one antimalarial drug are likely to be more useful than a technique which identifies a class, e.g. the 4-aminoquinolines, or is cross-sensitive to other commonly used medicines such as analgesics.

3. The threshold of detection of a test should be equivalent to, or better than, the minimum effective concentration of that drug or, if the lesser, the level at which that drug is known to cause adverse reactions when used in deliberate, or adventitious, combination with other drugs.
4. The reading of the test result should be rapid enough to permit its use for guidance when prescribing treatment in patients who have had, or may have had, previous medication, so as to avoid over dosage and combinations of contraindicated regimens.
5. The test should be adaptable to the widest possible range of test purposes such as screening of patients for drug trials, monitoring dosage and compliance, and guiding the choice of treatment and management of the disease.
6. If there are two, or more, competitive test systems, the one which best meets the most of the criteria for compliance, specificity, precision, cost and simplicity of use will be the logical candidate for adoption as a universal standard technique.

References

WHO (1984), Advances in malaria chemotherapy. Technical Report Series. Number 711. World Health Organisation, Geneva, Switzerland.

WHO (1989), Chemotherapy of malaria. Revised second edition. Monograph Series Number 27. World Health Organization, Geneva, Switzerland.

D.E. Davidson, MAP/RTI (1989). Personal communication. The half-life of halofantrine.

CHEMICAL ASSAYS FOR THE DETECTION OF ANTIMALARIAL DRUGS IN BODY FLUIDS

Frederick C. Churchill

Introduction

Methods for measuring antimalarial drug and metabolite concentrations in body fluids are important in many laboratory and field malaria studies and as a guide to decisions on treatment in the clinical setting. Results of these assays are used to screen patients for *in vivo* drug sensitivity studies and to verify ingestion and absorption of drugs. For *in vitro* field tests, researchers need to know if blood samples contain drugs that contribute to the antiparasitic effect of an added drug and produce false results. Assays may be used to monitor chemoprophylaxis and treatment compliance. Assays are also useful when exploring the relationship between use practices and incidence of disease, or the development of resistance in a community. Information on concentrations of antimalarial drugs in the blood of malaria patients to be treated can guide physicians in providing safe treatment.

The methods for detection and determination of antimalarials and their metabolites in body fluids have been reviewed recently, (Bergqvist & Churchill, 1988). Nonchromatographic and chromatographic methods of analysis were included but immunochemical assays were not discussed.

In our laboratory we have developed a 3-tiered approach to antimalarial drug assay. We classify assays as laboratory methods, field-interfaced laboratory methods, or field-adapted methods. Laboratory assays are those which require relatively sophisticated apparatus and techniques, and must be performed in a well equipped laboratory. Examples include methods for assay of chloroquine (CQ), (Bergqvist & Frisk-Holmberg, 1980; Alvan et al., 1982; Patchen et al., 1983), and mefloquine (MQ), (Bergqvist et al., 1988a; Bergqvist et al., 1988b). The most accepted reference method for CQ is high-performance liquid chromatography (HPLC), with fluorescence detection (Bergqvist & Frisk-Holmberg, 1980; Alvan et al., 1982; Patchen et al., 1983), due to its great sensitivity and selectivity and the ability to automate the procedure. Recently published methods for MQ employ HPLC with UV detection, (Bergqvist et al., 1988a), and GLC with derivatization and electron-capture detection, (Bergqvist et al., 1988b). Methods using such sophisticated analytical instrumentation have been interfaced with field studies by preserving finger-stick blood samples on filter paper to permit transport of the samples to the laboratory without refrigeration, (Patchen et al., 1983; Bergqvist et al., 1988c; Lindstrom et al., 1985; Bergqvist et al., 1987). These field-interfaced methods are available for CQ, (Patchen et al., 1983; Lindstrom et al., 1985), MQ, (Bergqvist et al., 1988b) and sulfadoxine, (Bergqvist et al., 1987).

Field-adapted Colorimetric Assays for CQ in Urine

It is often necessary, or desirable, to measure concentrations of chloroquine in urine, or blood, in the field and concurrent with ongoing studies. Compromises in selectivity, (specificity), sensitivity, and/or precision, are inevitable with field-adapted methods when compared to laboratory methods. Historically, colourimetric assays have been used for qualitative determination of CQ in urine in the field, (Wilson & Edeson, 1954; Haskins, 1958; Lelijveld & Kortman, 1970). A recent renaissance in colourimetric methodology for field-adapted detection of CQ and its metabolites, primarily DECQ, in urine, has included quantitative ion-pair extraction methods that use bromthymol blue, (Bergqvist et al., 1985), and methyl orange (Mount et al., 1987a); the Haskins test reagent.

All methods for field assay of CQ in urine developed before 1980 are qualitative, (Wilson & Edeson, 1954; Haskins, 1958; Lelijveld & Kortman, 1970). Because of its simplicity, the Dill-Glazko test, (Lelijveld & Kortman, 1970) has been most used in malaria field studies. However, recent publications report that this test is insufficiently sensitive, selective, and reliable for field use (Verdier et al., 1985; Rombo et al., 1985; Rombo et al., 1986). Sensitivity is variable; the detection limit of 40 to 80 mg/L depends on the pH of the urine, and haematuria can cause false positive results, (Rombo et al., 1986).

Three colourimetric methods - the bromothymol blue (BTB) method, (Bergqvist et al., 1985), and two modified Haskins methods, i.e. Haskins MMI and Haskins MMII, (Mount et al., 1987a), - permit quantification of CQ + metabolites in the field. Mount et al., (1987a) describe the use of a hand-held, battery-operated filter photometer, with 420-nm filter, in remote locations to facilitate colourimetric quantification of urinary CQ, (Fig. 1). Haskins MMI has a detection limit of 0.3 mg/L, and Haskins MMII, 1 mg/L. The BTB method can be modified to give a detection limit comparable to that of Haskins MMII (Y. Bergqvist, personal communication). Thus a negative result for any of these three tests corresponds to <1 mg/L CQ + metabolites in the urine. It has been proposed that this corresponds to less than 100 ug/L of CQ in the blood, the estimated minimal therapeutic concentration, (Mount et al., 1987b), so that these tests effectively screen patients with significant blood CQ levels to exclude them from drug sensitivity studies. The Haskins MMI assay requires more time and effort than the BTB or Haskins MMII procedures. Haskins MMII has been applied under field conditions to screen patients for an *in vivo* CQ sensitivity study, and to document ingestion and adsorption during the trial, (Steketee et al., 1987; Steketee et al., 1988). Positive results for CQ in urine show only moderate correlation with blood levels, (Bergqvist et al., 1985; Mount et al., 1987a; Steketee et al., 1988), so that analysis of selected, paired finger-stick blood samples by an HPLC-Fluorescence method in the laboratory can provide valuable additional information.

The method of E.G. Saker and E.T. Solomons for assay of drugs of abuse in urine, (Saker & Solomons, 1979), has been modified and adapted recently in our laboratory to permit facile semiquantitative, or quantitative, determination of CQ + metabolites in urine in the field, (Mount et al., 1989). Two methods have been validated and successfully applied. The simpler, semiquantitative method, designated S-S/CQI, has a detection limit of 1 ug/ml and is superior in every respect to the Dill-Glazko test in malaria field studies. The Table summarizes the characteristics of the various colourimetric field assays for CQ in urine.

The new semiquantitative and quantitative colourimetric methods for CQ + metabolites in urine, (Bergqvist et al., 1985; Mount et al., 1987a; Steketee et al., 1987; Steketee et al., 1988; Mount et al., 1989), can provide information on drug history to avoid toxic dosing in treatment, field screening to determine chemoprophylaxis and treatment compliance, and delineation of drug use practices. Reagents are stable, costs per test are five U.S. cents or less, and sampling throughout is rapid, (Churchill, 1989). The modified Haskins assays satisfy the "Land Rover Criterion" for field adaptability as shown in Fig. 2. Haskins MMI uses an aqueous defoamer, and Haskins MMII a glass-wool swab, to break emulsions without centrifugation. And both use a battery-operated filter photometer for CQ quantification so that no electricity is required. These methods, as well as the experimentally simpler S-S/CQI assay, have detection limits for CQ in urine of 1 mg/L or less. Typically CQ can be detected in the urine two weeks after a 300 mg as base prophylactic dose. S-S/CQI is being adapted to kit form in several formats to make this valuable tool more readily available to field workers at reasonable cost. A promising format for this test consists of the reagents sealed under vacuum in a small ampoule fitted with a small-diameter plastic tube at the narrow end. Breaking the ampoule at a pre-scored point under the tubing, while the end of the tubing is immersed in a urine sample, fills the tube with about 1 ml of urine. Shaking of the ampoule, and allowing separation of layers, permits the result to be read by eye and compared with standards if this is desired.

Prospects for Field-adapted Colourimetric Assays for Antimalarials Other Than CQ

The field-adapted colourimetric assays listed in Table 1 each show cross-reactivity with other drugs, including antimalarial drugs. The bromothymol blue, modified Haskins, and Saker-Solomons/CQ methods all give positive indications in the presence of quinine and/or proguanil. This is advantageous in screening for in-vivo drug sensitivity studies, where patients containing appreciable levels of any of these three drugs will be excluded from the trial on the basis of urine testing. If it is important to know the identity of the drug giving a positive

test, a more selective confirmatory test will be necessary. Because of the short half-lives of quinine and proguanil, monitoring of levels of these drugs, in many situations and types of studies, is less practical than for CQ.

The Bratton-Marshall test for sulfonamides (de Almeida-Filho & de Souza, 1983), has been used to screen for sulfadoxine in urine to verify history of Fansidar use. This test is nonspecific, giving positive tests for a wide variety of sulfonamides which might be encountered in the field setting. Interpretation of results can be subjective because of the observed colours, which are different shades, and tints, than the expected purple colour for a positive test. We feel that a test which employs partitioning of the diazo-coupled or other coloured product, into an organic phase could give a less ambiguous result. A confirmatory TLC test could be applied to samples giving a positive colourimetric result.

Work in our laboratory has shown that persons receiving 750 mg, as base, of MQ-HCl achieved urine concentrations of 0.5 to 2.0 mg/L MQ in urine, (unpublished results). Blood levels were in the range 0.40 to 1.2 mg/L in the first week after dosing. Not only are the urinary MQ concentrations low, but also they do not correlate particularly well with blood levels, indicating that pursuit of field assays for MQ in urine may not be a productive undertaking. Efforts to develop HPTLC and ELISA assays for MQ in blood appear to be more worthwhile for monitoring this drug in the field.

Conclusion

The topic of field-adapted assays for CQ and its metabolites in urine and blood, including colourimetric, high-performance thin-layer chromatographic (HPTLC), and enzyme-linked immunosorbent assays (ELISA), was reviewed earlier this year (Churchill, 1989). This workshop updated the information on CQ assays and extended these approaches to other antimalarial drugs, while evaluating the assay kits which are now available. Work accomplished to this point has demonstrated the value of immediately available analytical results to malaria field studies. It is important to consider the priorities for continued and future development, and the application of field-adapted assays for antimalarial drugs.

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References

- Alvan, G., Ekman, L. & Lindstrom, B. (1982). Determination of chloroquine and its desethyl metabolite in plasma, red blood cells and urine by liquid chromatography. Journal of Chromatography, 229, 241-247.
- Bergqvist, Y. & Frisk-Holmberg, M. (1980). Sensitive method for the determination of chloroquine and its metabolite desethylchloroquine in human plasma and urine by high-performance liquid chromatography. Journal of Chromatography, 221, 119-127.
- Bergqvist, Y., Hed, C., Funding, L. & Suther, A. (1985). Determination of chloroquine and its metabolites in urine: a field method based on ion-pair extraction. Bulletin of the World Health Organization, 63, 893-898.
- Bergqvist, Y., Hjelm, E. & Rombo, L. (1987). A sulphadoxine assay using capillary blood samples dried on filter paper suitable for monitoring of blood concentrations in the field. Therapeutic Drug Monitoring, 9, 203-207.
- Bergqvist, Y., Churchill, F.C. & Mount, D.L. (1988a). Determination of mefloquine by electron-capture gas-liquid chromatography after phosgene derivatization in biological samples and in capillary blood collected on filter paper. Journal of Chromatography, 428, 281-290.
- Bergqvist, Y., Hellgren, U. & Churchill, F.C. (1988b). High-performance liquid chromatographic assay for the simultaneous monitoring of mefloquine and its acid metabolite in biological samples using protein precipitation and ion-pair extraction. Journal of Chromatography, 432, 253-263.
- Bergqvist, Y. & Churchill, F.C. (1988c). Detection and determination of antimalarial drugs and their metabolites in body fluids. Journal of Chromatography, 434, 1-20.
- Churchill, F.C. (1989). Field-adapted assays for chloroquine and its metabolites in urine and blood. Parasitology Today, 5, 116, 121-126.
- De Almeida-Filho, J.M. & De Souza, J.M. (1983). A simple urine test for sulfonamides. Bulletin of the World Health Organization, 61, 167-168.
- Haskins, W.T. (1958). A simple qualitative test for chloroquine in urine. American Journal of Tropical Medicine and Hygiene, 7, 199-200.
- Lelijveld, J. & Kortman, H. (1970). The eosin colour test of Dill and Glazko: a simple field test to determine chloroquine in urine. Bulletin of the World Health Organization, 42, 477-479.
- Lindstrom, B., Ericsson, O., Alvan, G., Rombo, L., Ekman, L., Rais, M. & Sjogvist, F. (1985). Determination of chloroquine and its desethyl metabolite in blood: An application for samples collected in capillary tubes and dried on filter paper. Therapeutic Drug Monitoring, 7, 207-210.
- Mount, D.L., Patchen, L.C., Williams, S.B. & Churchill, F.C. (1987a). Colourimetric and thin-layer chromatographic methods for field assay of chloroquine and its metabolites in urine. Bulletin of the World Health Organization, 65, 615-623.
- Mount, D.L., Nahlen, B.L., Patchen, L.C. & Churchill, F.C. (1987b). Field-adapted method for high-performance thin-layer chromatographic detection and estimation of chloroquine and desethylchloroquine in urine. Journal of Chromatography, 423, 261-269.
- Mount, D.L., Nahlen, B.L., Patchen, L.C., Churchill, F.C. (1989). Adaptations of the Saker-Solomons test: simple reliable colourimetric field assays for chloroquine and its metabolites in urine. Bulletin of World Health Organization, 67(3): 295-300.
- Patchen, L.C., Schwartz, I.K., Mount, D.L. & Churchill, F.C. (1983). Analysis of filter-paper-absorbed, finger-stick blood samples for chloroquine and its major metabolite using high-performance liquid chromatography with fluorescence detection. Journal of Chromatography, 278, 81-89.
- Rombo, L., Bjorkman, A., Sego, E., Lindstrom, B., Ericsson, O. & Gustafsson, L.L. (1985). Reliability of Dill-Glazko test. Lancet, 1, 1509.
- Rombo, L., Bjorkman, A., Sego, E., Lindstrom, B., Ericsson, O. & Gustafsson, L.L. (1986). Evaluation of three qualitative tests for detection of chloroquine in urine - agreement with plasma concentration determined with liquid chromatography. Annals of Tropical Medicine and Parasitology, 80, 293-298.
- Saker, E.G. & Solomons, E.T. (1979). A rapid inexpensive presumptive test for phenacyclidine and certain other cross-reacting substances. Journal of Analytical Toxicology, 3, 220-221.
- Steketee, R.W., Brandling-Bennett, A.D., Kaseje, D.C.O., Schwartz, I.K. & Churchill, F.C. (1987). In-vivo response of Plasmodium falciparum to chloroquine in gravid and nulligravid women, Siaya District, Kenya. Bulletin of the World Health Organization, 65, 885-890.
- Steketee, R.W., Mount, D.L., Patchen, L.C., Williams, S.B., Churchill, F.C., Roberts, J.M., Kaseje, D.C.O. & Brandling-Bennett, A.D. (1988). Field application of a colorimetric method of assaying chloroquine and desethylchloroquine. Bulletin of the World Health Organization, 66, 485-490.

Verdier, F., Famanamirija, J.A., Pussard, E., Clavier, F., Biau, J.M., Coulanges, P. & Le Bras, J. (1985). Unreliability of Dill-Glaxo test in detecting chloroquine in urine. Lancet, 1, 1282-1283.

Wilson, T. & Edeson, J.F.B. (1954). Studies on the chemotherapy of malaria, III: The treatment of acute malaria with chloroquine. Medical Journal of Malaya, 9: 115-131.

Table 1

CHARACTERISTICS OF FIELD-ADAPTED COLOURIMETRIC ASSAYS
FOR CHLOROQUINE (CQ) IN URINE

Method	Detection Limit (mg/L)	Precision (%)	Selectivity (Specificity)	Compounds Detected
Wilson-Edeson	4	qualitative	fair	CQ, metabolites, quinine
Haskins	2	qualitative	fair	CQ, metabolites, quinine, proguanil
Dill-Glaxo	40 - 80 (variable)	qualitative	fair	CQ, AM, metabolites, quinine
Bromthymol Blue	3	5 - 10	fair	CQ, metabolites, quinine, proguanil
Haskins MM1	0.3	5 - 10	fair	CQ, metabolites, quinine, proguanil
Haskins MM11	1	5 - 10	fair	CQ, metabolites, quinine, proguanil
Saker-Solomons CQ1	1	qualitative	fair	CQ, metabolites, quinine, proguanil
Saker-Solomons CQ11	2	5 - 10	fair	CQ, metabolites, quinine, proguanil

Figure 1

HAND-HELD, BATTERY-OPERATED, FILTER PHOTOMETER FOR COLOURIMETRIC QUANTIFICATION

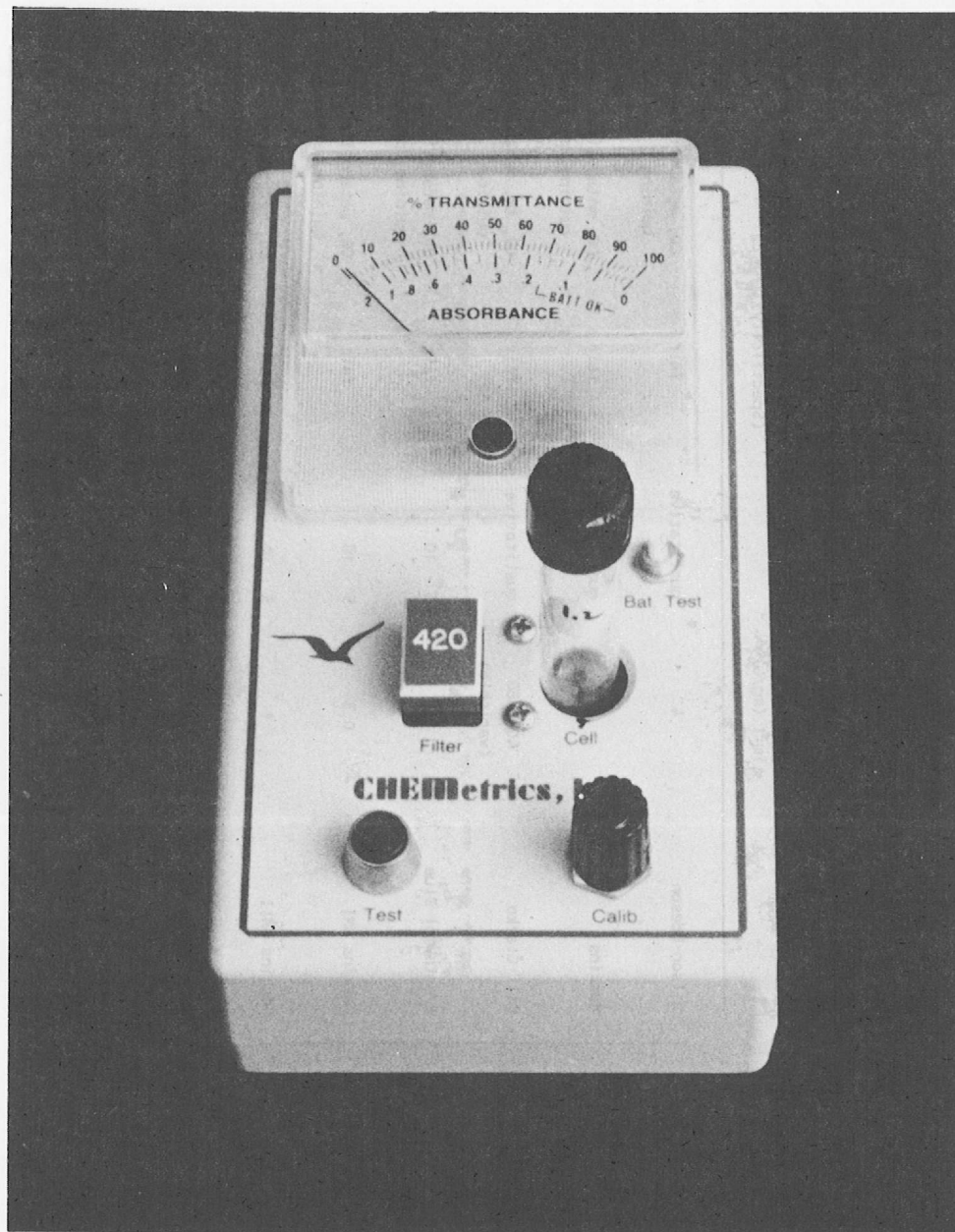


Figure 2

FIELD APPLICATION OF MODIFIED HASKINS ASSAYS IN STUDIES OF CQ EFFICACY IN MALARIA-INFECTED GRAVID AND NULLIGRAVID WOMEN IN THE SIAYA DISTRICT, WESTERN KENYA



Annex 1

SAKER-SOLOMONS, CQ MODIFICATION I TEST FOR CHLOROQUINE IN URINE

This test is adapted from a published method for phencyclidine (Saker and Solomons, 1979) based upon the extraction of chloroquine and its metabolites from urine into chloroform containing organic soluble tetrabromophenolphthalein ethyl ester (TBPEE). The extraction of the basic drug chloroquine effects a change in pH of the chloroform-TBPEE solution, causing colour change in this, lower layer from yellow-green to purple-red. The intensity of purple-red color is proportional to the concentration of chloroquine and metabolites in the urine. This test is primarily intended to be a qualitative test, but estimations can be made in the 0-3 mg/L or ppm range by comparing the appearance of the unknowns with samples spiked in the 0-3 mg/L or ppm range (Mount et al., 1989).

A. Preparation of Reagents

1. pH 8 phosphate buffer - dilute 162 g of $K_2HPO_4 \cdot 3H_2O$ and 5.0 g of KH_2PO_4 to 500 ml with water.
2. TBPEE solution - dissolve 50 mg of tetrabromophenolphthalein ethyl ester, which is available from Eastman Organic Chemicals, Rochester, NY, USA in 100 ml of chloroform in a 125-ml Erlenmeyer flask. Shake the chloroform solution with 10 ml of 2N HCl. After the phases are allowed to completely separate, as much as possible of the aqueous HCl phase is drawn off with a disposable Pasteur pipette. It should be possible to remove all but 1 or 2 ml of the aqueous layer. Store the TBPEE-in-chloroform solution under an added 10 ml of pH 8 phosphate buffer.

B. Preparation of Standards (Optional)

1. Stock standard - Chloroquine diphosphate, 5.0 mg as base, diluted with 5.0 ml of deionized water in a screw-cap vial.
2. Add each of the following volumes of the standard solution to a corresponding conical 15 ml glass graduated centrifuge tube using a 10 ul syringe.

Blank	0.0 ul
1 ppm (mg/L)	2.0 ul
2 ppm (mg/L)	4.0 ul
3 ppm (mg/L)	6.0 ul

The volumes shown above for each concentration are to be used whenever a 2 ml quantity of urine is taken for standards and samples as stipulated in the procedure.

C. Pre-charging of Tubes with pH 8.0 Buffer and TBPEE Solution

1. Add 1 ml of pH 8.0 buffer to each centrifuge tube being prepared for use.
2. Add 0.2 ml of 0.05% TBPEE to each tube.

D. Analysis of Samples

1. Add 2 ml of control, i.e. blank, urine to each standard, each of which contains added CQ, buffer, and TBPEE solution. Add 2 ml of each sample urine to a tube precharged with buffer and TBPEE solution.
2. Cap tubes and shake vigorously for 30 seconds.
3. Allow tubes to stand for about 10 minutes before reading result.
4. Estimations of CQ concentration can be made by comparing unknowns against standards.

E. Practical Considerations

1. Automatic pipettors with disposable tips are useful for charging the tubes with buffer and TBPEE solution. The same devices may be used to add the sample.
2. The limit of detection using the stated volumes of urine and reagents is 1 ppm, (mg/L).
3. Certain other drugs which are organic bases can give positive tests. Experience with field samples have indicated that such interferences are infrequent.

4. For samples which give a strong positive test, e.g. 3 mg/L, a smaller volume of urine sample may be used in a subsequent test to give a colour reaction in the readable range defined by the standards, e.g. 0 to 3 mg/L. The concentration for the sample may be roughly estimated by multiplying the ratio 2.0/volume taken x reading in mg/L from comparison with the standard series. For example, a 0.2 ml urine sample, which gives a result comparable to a 2 mg/L standard, contains roughly $2.0 \text{ ml} / 0.2 \text{ ml} \times 2 \text{ mg/L} = 20 \text{ mg/L}$ chloroquine.

F. References

Saker, E.G. and Solomons, E.T. (1979). A rapid inexpensive presumptive test for phenacycline and certain other cross-reacting substances. Journal of Analytical Toxicology, 3, 220-221.

Mount, D.L., Nahlen, B.L., Patchen, L.C. & Churchill, F.C. (1989). Adaptations of the Saker-Solomons test: simple, reliable colorimetric field assays for chloroquine and its metabolites in urine. Bulletin of the World Health Organization, 67, 295-300.

Note: A procedure is available, i.e. Saker-Solomons, CQ, Modification II, which permits quantification of CQ using a hand-held, battery-operated filter photometer.

Annex 2

SAKER-SOLOMONS, CQ, MODIFICATION II, TEST FOR CHLOROQUINE IN URINE

This test, adapted from a published method for phenacycline (Saker and Solomons, 1979) is based upon the extraction of chloroquine, and its metabolites, from urine into chloroform containing organic soluble tetrabromophenolphthalein ethyl ester (TBPEE). The extraction of the basic drug chloroquine effects a change in pH of the chloroform-TBPEE solution, causing a colour change in this, lower, layer from yellow-green to purple-red. The intensity of purple-red color is proportional to the concentration of chloroquine and metabolites in the urine to a concentration of about 8 ppm, (Absorbance = 0.5). For samples showing higher absorbance, a smaller volume of urine may be taken, the determination repeated, and the result calculated (Mount et al., 1989).

A. Preparation of Reagents

1. pH 8 phosphate buffer - dilute 162 g of $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$ and 5.0 g of KH_2PO_4 to 500 ml with water.
2. TBPEE solution - dissolve 20 mg of tetrabromophenolphthalein ethyl ester, which is available from Eastman Organic Chemicals, Rochester, NY, USA in 100 ml of chloroform in a 125-ml Erlenmeyer flask. Shake the chloroform solution with 10 ml of 2N HCl. After the phases are allowed to completely separate, as much as possible of the aqueous HCl phase is drawn off with a disposable Pasteur pipette. It should be possible to remove all but 1 or 2 ml of the aqueous layer. Store the TBPEE-in-chloroform solution under an added 10 ml of pH 8 phosphate buffer.

B. Preparation of Standards

1. Stock standard - Chloroquine diphosphate, 5.0 mg as base, diluted with 5.0 ml of deionized water in a screw-cap vial.
2. Add each of the following volumes of the standard solution to a corresponding 13 x 100 mm screw-cap culture tube using a 20 ul syringe.

Blank	0.0 ul
2 ppm (mg/L)	4.0 ul
4 ppm (mg/L)	8.0 ul
6 ppm (mg/L)	12.0 ul
8 ppm (mg/L)	16.0 ul

The volumes shown above for each concentration are to be used whenever a 2 ml quantity of urine is taken for standards and samples as stipulated in the procedure.

C. Pre-charging of Tubes with pH 8 Buffer and TBPEE Solution

1. Add 1 ml of pH 8 buffer to each culture tube being prepared for use.
2. Add 2.0 ml of 0.02% TBPEE to each tube.

D. Analysis of Samples

1. Add 2 ml of control, i.e. blank, urine to each standard, each of which contains added CQ, buffer, and TBPEE solution. Add 2 ml of each sample urine to a tube precharged with buffer and TBPEE solution.
2. Cap tubes and invert 30 times by hand.
3. Allow tubes to stand for at least 10 minutes.
4. Clarify the chloroform layer using a glass-wool swab.
5. Read percent transmittance of the chloroform layer using a hand-held, battery-operated filter photometer equipped with a 565-nm filter such as Chemetrics Inc., Calverton, VA, USA.
6. Record each value, convert to absorbance, and compare absorbance of samples to a standard curve constructed using the concomitantly extracted standards.

E. Practical Considerations

1. Automatic pipetters with disposable tips are useful for charging the tubes with buffer and TBPEE solution. The same devices may be used to add the sample.
2. The limit of detection using the stated volumes of urine and reagents is 2 ppm, or mg/L.

3. Certain other drugs which are organic bases can give positive tests. Experience with field samples have indicated that such interferences are infrequent.
4. A subsequent analysis can be performed on a smaller volume of urine. For samples giving a percent transmittance (% T) less than that for the 8 mg/L standard, and the appropriate factor included in calculation of the result.

F. References

- Saker, E.G. and Solomons, E.T. (1979). A rapid inexpensive presumptive test for phencyclidine and certain other cross-reacting substances. Journal of Analytical Toxicology, 3, 220-221.
- Mount, D.L., Nahlen, B.L., Patchen, L.C. & Churchill, F.C. (1989). Adaptations of the Saker-Solomons test: simple, reliable field assays for chloroquine and its metabolites in urine. Bulletin of the World Health Organization, 67, 295-300.

Note: A procedure is available, i.e. Saker-Solomons, CQ, Modification I, which permits estimation of CQ by visual comparison of samples with standards.

HASKINS, MOUNT MODIFICATION II METHOD FOR THE QUANTIFICATION OF CHLOROQUINE AND METABOLITES IN URINE UNDER FIELD CONDITIONS

The Haskins, Mount Modification II, Haskins MMII, method for quantification of Chloroquine (CQ), and metabolites in urine is based on ion-pair formation between CQ and methyl orange in chloroform (Haskins, 1958). Values for percent transmittance (% T), are read for standards and samples using a hand-held, battery-operated filter photometer, and are converted to absorbed values which are linearly related to chloroquine concentrations in urine up to 32 mg/L or ppm. The detection limit for the method is 1 mg/L, or ppm. For concentrations up to 10 mg/L, the % T value may be read directly from the filter photometer. For samples with concentrations between 10 and 32 mg/L, one may either dilute the chloroform layer before reading % T, or take a smaller urine sample and rerun the test, including extraction. For samples containing greater than 32 mg/L, a correspondingly smaller urine sample must be taken to assure that the method is being employed in the linear range. Although the method measures primarily CQ, there is also some contribution to the final result from desethylchloroquine (DECQ), the major metabolite of CQ.

A. Preparation of Reagents

1. pH 8 phosphate buffer - dilute 81 g of $K_2HPO_4 \cdot 3H_2O$ and 2.5 g of KH_2PO_4 to 250 ml with water.
2. Methyl orange solution, i.e. nominal 0.2% methyl orange in 5% aqueous boric acid - dilute 0.5 g of methyl orange and 12.5 g of boric acid (H_3BO_3), to 250 ml with water. Allow the mixture to stand for at least four hours with occasional agitation. Filter the mixture through a medium porosity, fluted filter paper.

B. Preparation of Standards

Stocks standard - Chloroquine diphosphate, 5.0 mg as base, diluted with 5.0 ml of deionized water in a screw-cap vial.

Add the following volumes of the standard solution to 13 mm O.D. x 100 mm culture tubes to give the corresponding standards:

Blank	0.0 ul
1.5 ppm (mg/L)	3.0 ul
3.0 ppm (mg/L)	6.0 ul
6.0 ppm (mg/L)	12.0 ul
10.0 ppm (mg/L)	20.0 ul

If a 10 ul syringe is used, the 6.0 ppm standard may be made with two additions of 6.0 ul each and the 10.0 ppm standard with two additions of 10.0 ul each. The volumes added for each concentration are to be used whenever a 2 ml quantity of urine is taken for standards and samples, as stipulated in the procedure.

C. Pre-charging of Tubes with pH 8 Buffer and Chloroform

1. Add 1 ml of pH 8 buffer to each culture tube being prepared for use.
2. Add 2 ml of chloroform to each culture tube.

D. Analysis of Samples

1. Add 2 ml of control, i.e. blank, urine to each standard, each of which contains added CQ, buffer, and chloroform. Add 2 ml of each sample urine to culture tubes precharged with buffer and chloroform.
2. Cap, invert the culture tubes 20 times, and allow the phases to separate. Many tubes may be grasped and inverted simultaneously.
3. Draw off the majority of the aqueous urine phase with a Pasteur pipette and add 1 ml of 0.2% methyl orange in aqueous 5.0% boric acid.
4. Cap, invert 20 times, and allow phases to separate; use a glass-wool swab to clarify the chloroform layer.
5. Place each culture tube in succession in a battery-operated, filter photometer containing a 420 nm filter. Rotate the tube slowly to the position giving a maximum % T (minimum absorbance). Read % T, convert to absorbance, and calculate CQ concentration from a standard curve.

E. Practical Considerations and Discussion

1. Automatic pipettors with disposable tips are useful for charging the tube with buffer and then with chloroform. These same devices may be used to add samples, changing tips for each sample, and the methyl orange solution.

2. To conserve glassware, the same Pasteur pipette may be used to separate the urine phase for a series of standards, and samples, being run.
3. The glass-wool swab is revolved, and rotated, in the chloroform phase to clear this phase. Excess liquid is expressed by pressing the swab to the side of the culture tube above the level of the aqueous layer. When two serial rinses in water are employed, a given swab may be used for a series of standards, and samples, without the occurrence of cross contamination.
4. A reliable and inexpensive, battery-operated, filter photometer for use in this work is the Model A-1050 available from Chemetrics, Inc. of Calverton, VA, USA. This filter photometer, equipped with batteries and a 420 mm filter, costs about \$340.
5. Rotate the 13 mm O.D. tubes to give a maximum % T and minimize the contribution of droplets of aqueous methyl orange solution which occasionally adhere to the inside of the tubes at the level where the light transmission is being measured.
6. A % T reading of 70% in the Haskins, MMI test corresponds to a CQ + metabolites concentration of about 1 mg/L in urine. In practice, 70% is the value below which the test is considered positive. Samples with a % T > 70% have < 1 mg/L CQ + metabolites in urine, which, as a general rule, corresponds to < 100 ug/L CQ in blood.

F. Example of a Standard Curve

The filter photometer is set to 100% transmittance with chloroform in a culture tube using the calibration adjustment.

Tubes containing samples and standards are rotated in the filter photometer to maximize the % T reading; this is the value taken for % T.

Data for standard curve:

Concentration of Standard (ppm)	% T	A
Blank (0.0 ul of 1.0 mg/L)	92	0.0362
1.5 (3.0 ul of 1.0 mg/L)	65	0.187
3.0 (6.0 ul of 1.0 mg/L)	46	0.337
6.0 (12.0 ul of 1.0 mg/L)	21	0.678
10.0 (20.0 ul of 1.0 mg/L)	08	1.110

$$Y = 0.1081X + 0.0264; r = 0.9998$$

It is recommended that a 3.0 ppm standard be freshly made and extracted each working day. The original standards should be read using the filter photometer and the newly made 3.0 ppm standard compared with the original 3.0 ppm standard. If the % T reading for the newly made 3.0 ppm standard varies by as much as 10% relative difference from the original standard, a new set of standards should be processed, and a new standard curve calculated. Standards and samples should always be processed using the same methyl orange solution. Experience has indicated that a given methyl orange solution may be used for at least one week before recalibration becomes necessary.

G. Reference

Haskins, W.T. (1958). A simple qualitative test for chloroquine in urine. American Journal of Tropical Medicine and Hygiene, 7, 199-200.

VALIDATION OF THE SAKER-SOLOMONS CQI AND HASKINS MMII COLOURIMETRIC TESTS FOR FIELD ASSAY OF CHLOROQUINE IN URINE

Objective

To evaluate the results of the analysis of urine samples for chloroquine by the Saker-Solomons CQI and Haskins MMII colourimetric methods, in comparison to patient history and concentrations found by a reference HPLC method.

Experimental

Twenty urine samples labeled C-1 through C-20 were analyzed, first by the Saker-Solomons CQI method and then by the Haskins MMII method. The Saker-Solomons assay was applied to the samples as described in Annex 1. The Haskins MMII assay was applied to the samples as described in Annex 3, except that different concentrations were chosen for the standards.

Results

Results of application of the Haskins MMII method to samples C-1 through C-20 are shown in Table 1, and include standard-curve data.

Results of application of the Saker-Solomons CQI test, and the Haskins MMII test to samples C-1 through C-20, are compared in Table 2 with those from the reference HPLC method.

Discussion

In general the Saker-Solomons CQI and Haskins MMII results agreed well with one another and with the HPLC values. Quinine and mefloquine also give positive Saker-Solomons CQI and Haskins MMII tests, with sensitivities comparable to that for CQ. Generally MQ concentrations in urine are low, e.g. a few mg/L, even when blood concentrations are as great as 1000 to 1500 mg/L. Sample C-6 contained MQ.

For Sample C-13 there was a question as to whether the same sample was provided for both the Saker-Solomons CQI and the Haskins MMII test. Samples C-13 and B-15 were allegedly subsamples of the same original sample. B-15 gave a Haskins MMII test result of 3.74 mg/L, while C-13 yielded 0.99 mg/L by this test. This discrepancy is confounded by the discovery that subsamples B-19 and C-20, allegedly from the same sample, were different, as shown by colourimetric and TLC tests.

The inconsistency in results for samples C-17 is addressed in the footnote to Table 2.

Conclusion

The Saker-Solomons CQI and Haskins MMII assays for CQ in urine are useful in screening patients to determine CQ status in a field setting. Thin-layer chromatographic methods can provide confirmation of positive tests if need for this information justifies the additional effort. If it is required, a concurrently taken filter-paper-preserved, finger-stick blood spot can be set aside and analyzed later by HPLC.

Table 1

RESULTS OF ANALYSIS OF SAMPLES C-1 THROUGH C-20
FOR CHLOROQUINE BY THE HASKINS MMII ASSAY

Sample of Std. Designation	Transmittance %	Absorbance (units)	Conc. Found (mg/L)
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Standard Curve

0.00 mg/L	72.0	0.143	0.30
2.79 mg/L	46.0	0.337	2.35
6.16 mg/L	20.0	0.699	6.18
9.24 mg/L	10.0	1.00	9.36

$$y = 0.0946x + 0.1146; r = 0.997$$

Sample Analysis

C-1	76.0	0.119	0.046 (<1)
C-2	0	high	>9
C-3	0	high	>9
C-4	10.0	1.0	9.36
C-5	77.0	0.114	0.0 (<1)
C-6	66.0	0.180	0.69 (<1)
C-7	76.0	0.119	0.046 (<1)
C-8	53.0	0.275	1.70
C-9	36.0	0.444	3.48
C-10	0	high	>9
C-11	3.0	0.523	4.32
C-12	72.0	0.143	0.30 (<1)
C-13	62.0	0.208	0.99
C-14	76.0	0.119	0.046 (<1)
C-15	74.0	0.131	0.173 (<1)
C-16	0	high	>9
C-17	33.0	0.481	3.87
C-18	11.5	0.939	8.72
C-19	29.5	0.530	4.39
C-20	77.0	0.114	0.0 (<1)

Table 2

COMPARISON OF SAKER-SOLOMONS CQI AND HASKINS MMII RESULTS
WITH THOSE FROM THE REFERENCE HPLC METHOD

Sample No.	S-S CQI (mg/L)	Haskins MMII (mg/L)	CQ by HPLC (mg/L)	Other Drugs Present
C-1	<1	<1	-	Quinine
C-2	>3	>9	-	Quinine
C-3	>3	>9	-	Quinine
C-4	>1	9.5	-	-
C-5	<1	<1	-	Mefloquine
C-6	2-3	0.69	-	Mefloquine
C-7	<1	0.05	-	FANSIMEF
C-8	3-4	1.71	-	FANSIMEF
C-9	3	3.48	3.8	-
C-10	>3	>9	12.6	-
C-11	>3	>9	15.8	-
C-12	1	0.30	-	Mefloquine
C-13	>3	0.99	-	Mefloquine
C-14	1	<1	-	-
C-15	1-2	<1	-	-
C-16	>3	>9	15.9	-
C-17	a	3.87	3.9	-
C-18	>3	8.72	13.1	-
C-19	>3	4.39	6.0	-
C-20	1	<1	-	-

- a) The Saker-Solomons CQI test gave an atypical blue colour. It is suspected that a compound other than CQ may be present which also gives a positive result for the Haskins MMII assay.

SUMMARY OF FINDINGS FOR COLOURIMETRIC METHODS

These discussions have centered on three possible applications of this methodology which, it is felt, could replace the widely used Dill-Glazko test as the basic field test for the detection of chloroquine in urine.

The Dill-Glazko test has gained wide acceptance due to its simplicity and low cost. However, recent research has shown that it has low sensitivity and reliability, [Rombo et al., 1986].

The three methods evaluated were Saker-Solomons CDC CQI, in two formats, and Haskins MMII.

1. Saker-Solomons CDC CQI

This test is as easy to run as the Dill-Glazko, and provides superior sensitivity and reliability, i.e. detects 1 mg/L in urine. It is applicable to the whole range of operational uses and research: premedication studies; post medication studies; drug distribution in the community and research applications. However, its use at the laboratory level is probably not justified as other methods, such as HPLC, TLC, HPTLC, and ELISA, have higher sensitivity and reliability.

The development of the Saker-Solomons CDC CQI test is complete and only needs a centralized supply system to be established. However, such is the simplicity of the kit preparation, and the general availability of the components for the kit, that localized, regional, distribution is a feasible and, perhaps, preferable system of distribution.

Apart from the obvious and known toxic nature of the component chloroform, there is little danger of misadventure in the routine, normal, and controlled use of the kits.

The detection limit of the test automatically excludes its possible use as a routine test for antimalarials in other body fluids such as whole blood, plasma, and serum.

The extent of training required is minimal: microscopist level personnel could be satisfactorily trained in two days.

2. Haskins MMII Test

This is a test which, by comparison with the Saker-Solomons, provides a more quantitative analysis at the expense of modestly increased complexity and cost.

The detection limit, i.e., 1 mg/L, is the same as for the Saker-Solomons CQI assay as is the range of operational research applications. Alternative, more highly selective and sensitive test procedures, i.e., HPLC, would be used at laboratory level.

The status of development is complete and, again, a decentralized supply system appears feasible provided an appropriate version of the photometer is available, or procurable, at the local level.

The toxicity considerations are the same as for the Saker-Solomons tests except that the volume of chloroform which has to be disposed of in a safe manner is considerably greater, i.e., a series of 100 tests yields 200 ml chloroform.

The training requirements are somewhat enhanced, four days being required for microscopist level personnel.

Recommendations

1. A study is required to determine the correlation between urinary, whole blood and plasma concentrations of chloroquine.

This study would involve paired sampling of serial collections from selected cases.

Urine would be analyzed for chloroquine and creatinine, and chloroquine concentrations would be normalized in relation to creatinine concentrations.

The normalized, corrected, values would be paired to chloroquine concentrations in whole blood and plasma to determine the predictive value of the urinary values relative to whole blood and plasma concentrations. A protocol is being prepared to conduct the necessary experiments.

2. A proposal should be formulated to establish, as soon as possible, an inter-regional training course for trainers to disseminate the procedures required to determine concentrations of antimalarial drugs in body fluids.

The training should be oriented to the promotion of the transfer of methodology of field adapted assays to the widest possible extent.

3. Efforts should be made to establish a distribution system for the colourimetric assays, which will ensure the availability of test material when the trained personnel become available at the operational level.

Reference

1. Rombo, L., Bjorkman, A., Sego, E., Lindstrom, B., Ericsson, O. and Gustafsson, L.L. (1986). Evaluation of three qualitative tests for detection of chloroquine in urine - Agreement with plasma concentrations determined with liquid chromatography. Annals of Tropical Medicine and Parasitology, 80, 293-298.

PRODUCTION OF MONOCLONAL ANTIBODIES AGAINST ANTIMALARIAL DRUGS FOR USE IN IMMUNOASSAYS

Teunis A. Eggelte

Introduction

Drug resistant Plasmodium falciparum malaria is an increasing problem in malaria control; especially the resistance of P. falciparum to chloroquine, (Bruce-Chwatt, 1982; Onori, 1984) since chloroquine was, and in many cases still is, the drug of choice for both treatment and preventive of malaria, (Cook, 1988). Chloroquine has been highly effective, inexpensive and has had relatively few side effects and when chloroquine needs to be replaced by other antimalarials, it will markedly increase the costs in malaria control (Spencer, 1985). A complicating factor is that there is no alternative antimalarial which can replace chloroquine in all its applications, and that resistance of P. falciparum to other antimalarial drugs is also increasing, (Peters, 1987).

A report of drug resistant malaria in a particular country does not necessarily imply that a certain drug, e.g. chloroquine, can no longer be used. There may be large differences in the geographical distribution of the drug resistance, and information about the spread of drug resistant P. falciparum is urgently needed. Such information can be obtained through in vivo and in vitro drug susceptibility studies of P. falciparum. Subjects selected for both in vivo and in vitro testing should not have a history of recent drug intake. Antimalarial drugs are, in most malaria endemic areas, freely available on the local market, and their use is presumed to be widespread. However, there is hardly any information available on drug utilization in a population and there is no insight into the drug pressure encountered by the parasites. This drug pressure is most likely a contributing factor in the process of the development of resistance. Failure of radical cure, or failure of chemosuppression, may not always be the result of the presence of drug resistant parasites, it can also be due to insufficient blood drug levels.

There is, therefore, a need for tests which can detect antimalarial drugs in biological fluids.

Tests for antimalarial drugs can be used in:

- in vivo drug resistance studies
- in vitro drug resistance studies
- drug compliance studies
- drug utilization studies
- pharmacokinetic studies
- basic research

Excellent methods for the determination of drugs in body fluids are available such as HPLC techniques, (Bergqvist, 1988). However these techniques are expensive, laborious and require sophisticated instrumentation.

New tests need to be:

- sensitive
- specific

When used under field conditions, e.g. a simple field laboratory, a test should have, ideally, the further requirements of:

- simple assay procedure
- simple instrumentation
- small sample volumes
- stable reagents
- low cost
- timing that fits work/treatment patterns

Tests based on immunochemical techniques can fulfil many of these requirements. Specificity may be ensured by the characteristics of the antibodies, and immunoassays can be developed over a wide range of levels of technology

The type of test required will be influenced by the matrix (body fluid), to be analyzed, and whether a qualitative or quantitative answer is needed. Drug concentrations in urine are in general, much higher than those in blood and, therefore, less sensitive tests may be used. In the case of qualitative tests it can be useful to look at a metabolite of the drug rather than the parent drug, since the former may be present in higher concentrations in the urine.

The choice of the samples to be collected and analyzed will depend on a number of factors. For socio-cultural reasons collection of urine samples may sometimes be impossible, and collection of urine young children is always rather difficult. In most instances venous blood is impossible to obtain. However collection of finger prick blood will, in general, be more acceptable, especially in connection with malaria diagnosis. The sensitivity of the test should be governed by the type of sample to be analyzed. Whole blood samples may be preferred to plasma samples, as the drug concentration will depend upon the quality of the plasma, e.g. haemolysis.

Development of Immunoassays for Antimalarial Drugs

A key in the development of immunoassays for antimalarial drugs is the production of specific antibodies against these drugs. To obtain antibodies against antimalarial drugs, we have relied a great deal on the use of the hybridoma technology to produce monoclonal antibodies. However polyclonal rabbit antisera have also been prepared against some drugs.

The advantages of monoclonal antibodies are:

- indefinite supply of antibody(ies) with constant characteristics
- affinity, and fine specificity, are defined and may be selected to suit applications
- easy purification in large quantities which do not damage immunoreactivity
- clean reagents giving low non-specific binding and background

For applications in certain types of immunoassays such as agglutination assays, the use of polyclonal antibodies will be more suitable than that of monoclonal antibodies, which react only with one epitope. However, the use of more than one monoclonal antibody, with different specificities or hybrid antibodies, may overcome this problem. In the case of the development of agglutination inhibition assays for drugs, this will be no problem as, generally, use is made of multivalent drug antigens.

In a programme to develop immunoassays for the whole range of antimalarial drugs, we have produced, so far, polyclonal and/or monoclonal antibodies against the following antimalarials:

chloroquine	sulfadoxime	(chlor)proguanil
quinine	dapsone	(chlor)cycloguanil
mefloquine	pyrimethamine	

Production of antibodies against other antimalarials is being planned.

These antibodies are used in the development of competitive binding immunoassays, especially in ELISA inhibition tests for these drugs. An important aspect of the production of antibodies against drugs, e.g. antimalarials, is the preparation of the drug-protein conjugate to be used for the immunization of mice and rabbits. The site of attachment of the linker between the drug and the protein, will determine to a large extent the specificity of the antibodies. The specificity of a polyclonal antiserum, containing many different antibodies with varying affinities and specificities, is something which cannot be selected, although immunization of more animals of the same species or other species may provide antisera with different characteristics.

Production of monoclonal antibodies may provide antibodies with different fine specificities. Furthermore, antibodies may be selected which have particular properties such as monoclonal antibodies with a special immunoglobulin sub-class type.

For the production of monoclonal antibodies against the various antimalarials, we fused spleen cells of immunized balb/c mice with NS-2 or Sp2/O myeloma cells. The fusion mixture was divided over ten microtitre plates and hybridoma supernatants screened, after 12 days, for presence of specific antibodies using an ELISA system. This system is similar to the one used in the indirect ELISA inhibition tests for the antimalarial drugs. In these tests drug antigens are coated onto polystyrene solid phase of a 96-wells microtitre plate, and the drug in solution competes with the solid phase drug for binding with specific antibody, (Fig. 1). Binding of antibody is detected using an enzyme labeled second antibody. Decrease in colour formation indicates the presence of drug in the assay system. Positive wells were rescreened with, and without, the presence of the drug. When full inhibition is seen in presence of the drug, it is a confirmation of the specificity of the drug antibodies. The coatings antigen used in the ELISA can either be the immunogen itself or, preferably, a conjugate of the drug with a different protein or a drug-polymer conjugate. After initial selection of the "clones", these are recloned thrice to ensure monoclonality. For the production of ascites fluid, the hybridomas are injected intraperitoneally into the balb/c mice.

Monoclonal Antibodies Against Antimalarial Drugs

Chloroquine

For the immunization of balb/c mice, and the preparation of coating antigens for the ELISA, a number of different chloroquine-protein, e.g. BSA or HCH, conjugates have been produced. In our first approach to produce monoclonal and polyclonal antibodies against chloroquine, we coupled hydroxychloroquine plaquenil, to BSA by converting the hydroxy group into a methanesulphonyl group, and coupling the mesylate to the protein, (Fig. 2). Another method of coupling hydroxychloroquine to a protein was by reaction of hydroxychloroquine with dinitrophenylchloroformate and adding the reaction product to the protein. From our fusion F73 we obtained 6 monoclonal antibodies against chloroquine, which were of the IgG1 and IgG2b sub-class. Other research groups, which have produced polyclonal and/or monoclonal antibodies against chloroquine, used drug-protein conjugates prepared from bisdesethylchloroquine, (Rowell & Rowell, 1987), and 4-amino-7-chloroquinoline, (Freier et al., 1986a, 1986b).

The antibodies produced against these types of chloroquine-protein conjugates do not only react with chloroquine but also with metabolites of chloroquine such as desethylchloroquine and amodiaquine. The reaction of monoclonal antibodies of fusion F73 with desethylchloroquine is similar to that of chloroquine, and cross reactivity of amodiaquine is about 5-10%. The reason for this cross reactivity is that the antibodies are directed against the 4-amino-7-chloroquinoline part of the chloroquine molecule. When we looked at the reactivity of a chloroquine analogue lacking the

chloro-substituent at the 7-position, we found that almost all reactivity was abolished. Based on information obtained on the reactivity of monoclonal antibodies F73-1 and F73-8, with different chloroquine analogues, we use a polyacrylamide polymer, containing 4-(3-aminopropylamino)-7-chloroquinoline groups, as coating antigen (fig. 3) in the ELISA for chloroquine. This antigen can be prepared by copolymerization of the two acrylamide components, or by reaction of 4-(3-aminopropylamino)-7-chloroquinoline with a acrylamide-acrylic ester copolymer containing N-hydroxysuccinimidyl ester groups. Other poly-acrylamide chloroquine coatings antigens can be prepared by copolymerization of acrylamide with the acrylester of hydroxychloroquine.

More specific antibodies against chloroquine can be prepared by using a chloroquine-protein immunogen in which chloroquine is linked at a ring position to the carrier protein. Several of such antigens have been synthesized. We have been successful in producing monoclonal antibodies against a 7-(3-aminopropylamino)-4-(4-diethylamino-1-methylbutylamino)-quinoline protein conjugate, (Fig.4). This approach is more or less similar to that used by Rowell & Rowell, (1987). A number, (11), monoclonal antibodies were obtained. The antibodies of fusion F149 do not only react with antigens derived from 7-(3-aminopropylamino)-4-(4-diethylamino-1-methylbutylamino)-quinoline, such as the immunogen and poly-acrylamide type antigens, but also with antigens derived from 8-aminochloroquine, such as a 8-diazochloroquine-BSA conjugate and a copolymer of acrylamide, and the acrylamide of 8-aminochloroquine, (Fig. 5).

The monoclonals show no, or hardly any, cross reactivity with amodiaquine, and react with desethylchloroquine to a much lesser extent than monoclonals F73-1 and F73-8.

Using the antibodies F73-1 or F73-8, indirect ELISA inhibition tests for chloroquine can be developed which have an in assay sensitivity of around 2 ug/L, (50% inhibition). Also with monoclonal F149012, we can obtain about the same order of sensitivity. Different peroxidase substrates can be used such as 5-aminosalicylic acid, (5-AS), 3,5,3',5'-tetramethylbenzidine, (TMBD), or ortho phenylenediamine, (OPD). As sensitivity of the ELISA inhibition tests is a function of coatings antigen and antibody concentration, the use of a more chromogenic substrate will improve the sensitivity of the tests, since it allows the lower coatings concentrations and/or higher antibody dilutions.

The procedure of the ELISA can be simplified by using directly labelled antibodies, thereby omitting the step of incubation with enzyme labelled second antibodies, (Fig. 6).

The sensitivity of the direct test is about the same as the indirect test.

Using the monoclonal antibodies against chloroquine, a different type of inhibition test can also be developed. In this new approach competition between solid phase bound drug, and drug in solution for specific antibody, is replaced by competition of drug and enzyme labelled drug for binding to solid phase bound specific antibody (fig. 7). For this approach 4-(3-aminopropylamino)-7-quinoline and 7-(3-aminopropylamino)-4-diethylamino-1-methylbutylamino) quinoline have been coupled to horse radish peroxidase, (HRP), (Fig. 8).

Using these "chloroquine"-HRP conjugates, tests can be set up which have similar sensitivities as those described for the other ELISA's. An advantage of using the enzyme labelled drugs is the ease of preparation compared with the labelling of antibodies.

Quinine

Due to the increasing resistance of *P. falciparum* to chloroquine, quinine is at the moment the drug of choice in the management of severe malaria.

Although many tests for quinidine are commercially available, this is not the case for quinine. However, there are several reports on the production of antibodies against quinine, and use of these antibodies in immunoassays, (Morgan et al., 1985; Sidki et al., 1987a, 1987b; Ward & Morgan, 1988; Eslava et al., 1988). For the production of antibodies against quinine, the method mostly used to couple quinine to a protein is to introduce a succinyl linker at the hydroxy group at the 9-position. We have used this conjugate for immunization of mice, but for the preparation of one of our quinine coatings, we use antigens by copolymerization of acrylamide and the vinyl group of the quinuclidine part of the quinine molecule.

For the production of monoclonal antibodies against quinine we also used a 9-hemisuccinylquinine-BSA conjugate, (Fig. 9). A series of 11 monoclonal antibodies were obtained, and tested for their specificity, by looking at their cross-reactivity with other cinchona alkaloids. Cross-reactivity of our monoclonal antibodies with quinidine is low, ranging from 0.01 - 0.5%, (Fig. 10). The highest reactivity is seen with dihydroquinine, (20-50%); cinchonine also reacted with all the monoclonal antibodies, (5-20%).

As may be expected from the low reactivity of quinidine, cinchonine showed only very low reaction, (< 0.01%), with the monoclonal antibodies. Surprisingly, when we tested the reactivity of a 1:1 mixture of epi-quinine and epi-quinidine, (unnatural isomers of quinine and quinidine), some of the monoclonals showed high reactivity whereas others showed only low. As the contribution of epi-quinidine will be probably negligible, epi-quinine reacts in 50-100% with some of these monoclonal antibodies against quinine, thereby illustrating the different fine specificity of various monoclonal antibodies.

When we use a BSA-succinylquinine coatings antigen, or a coatings antigen prepared by copolymerization of acrylamide with 9-0-acryloyl quinine in combination with monoclonal F125-1, we can obtain a sensitivity of 10 ug/L (50% inhibition). When instead of a quinine coatings antigen, we use a coatings antigen based on cinchonidine, the sensitivity for quinine can be improved.

The same idea of influencing the sensitivity, by varying the affinity of the antibody for the solid phase antigen, can also be applied on coatings antigens on the basis of co-polymers of acrylamide and quinine, which resembles the dihydroquinine structure. In this case, we see also an improvement of the sensitivity.

Also, for quinine, a test can be developed on the basis of enzyme labelled quinine and solid phase bound quinine antibodies. The sensitivity of this type of assay is similar to that using solid phase bound quinine.

Mefloquine

Mefloquine is increasingly used in the treatment of malaria as a replacement for chloroquine in areas where chloroquine resistance of *P. falciparum* is extensive. Use of mefloquine for malaria chemoprophylaxis is also rising. Moreover, the side effects of treatment with mefloquine are being reported with increasing frequency.

The author knows of no publications concerning the production of antibodies against mefloquine. For the preparation of a mefloquine-protein conjugate we reacted mefloquine with succinic anhydride to introduce a succinyl linker on the nitrogen of the piperidine ring, (Fig. 11).

After a number of attempts we finally obtained a series of monoclonal antibodies which showed excellent properties. These antibodies can be used in combination with different coating antigens, which are prepared by reaction of mefloquine with acryloyl chloride, vinylchloroformate, or divinylsulphone, and co-polymerization of the reaction products with acrylamide, (Fig. 12). The sensitivity which can be obtained under critical conditions, is in the order of 1-2 ug/L. An important property of these antibodies is their lack of reaction with the major metabolite of mefloquine, which is present in the blood in a much higher concentration than mefloquine itself.

Using the same manner as was employed for the immunization antigen of mefloquine, mefloquine could also be coupled to horse radish peroxidase. Preliminary results show that the ELISA using these labelled antibodies, has a similar sensitivity to that of ELISA using labeled antibodies.

Use of Monoclonal Antibodies in Tests for Antimalarial Drugs

Work is in progress to develop different types of immunoassays for a whole series of antimalarial drugs and to standardize these tests so that kits can be produced for these antimalarial drugs.

An advantage of the use of immunoassays for demonstration and determination of drugs in body fluids, is that no sample pretreatment is necessary except for making a dilution of the sample. Furthermore, many samples can be tested simultaneously. Immunoassays are, therefore, excellent methods for handling the large numbers of samples which are necessary in epidemiological studies. Different types of tests have to be developed so that not only large batches of samples can be handled, but also so individual samples can be tested when a quick answer is required. In the last few years tests have come onto the market which are intended for "in office" and "in home" use (Auditore-Hargreaves et al., 1988; May, 1988). Examples are the whole range of pregnancy tests and "on site" tests (Chesham & Anderton, 1988), in the field of veterinary diagnosis. Also many types of simple HIV tests have been developed. Some of these new technologies may be also used for the development of simple tests for the detection of antimalarial drugs. The tests described in this paper are all based on the principle of competition and the absence of, or reduction in, colour formation as an indication for the presence of a particular drug in the sample. It may be possible also to develop tests which give a positive signal (colour formation), when the drug is present.

One can imagine the development of tests whereby more than one antimalarial drug can be detected simultaneously, using tests strips containing several spots with different coatings of antigens or specific antibodies.

A number of ELISA tests for antimalarial drugs have been used to detect these drugs in the urine, or the blood, of malaria patients, (Greenwood et al., 1986; Shenton et al., 1988; Eggelte et al., 1988; Van der Kaay et al., 1988). Tests for dapsone and pyrimethamine have been used in chemoprophylaxis compliance studies and tests for chloroquine and sulfadoxine in connection with in vivo and in vitro drug resistance studies. By testing of urine samples collected from patients seeking treatment for malaria in various hospitals in Kenya, Tanzania, Malawi, Zambia and Ghana, results were obtained which showed that the patients had often already taken chloroquine. A percentage of 50%, or even higher, was not unusual. The percentage of positives among malaria patients was much higher than that found in patients coming to the hospital with other complaints.

References

- Auditore-Hargreaves, K. & Hargreaves W.R. (1988). In W.P. Collins Complementary Immunoassays, John Wiley & Sons.
- Bergqvist, Y. (1988). Detection and determination of antimalarial drugs and their metabolites in body fluids. Journal of Chromatography, 434, 1-20.
- Bruce-Cchwatt, L.J. (1982). Chemoprophylaxis of malaria in Africa: the spent "magic bullet". British Medical Journal, 285, 674-676.
- Chesham, J. & Anderton, D.J. (1988). In W.P. Collins, Complementary Immunoassays, John Wiley & Sons.
- Cook, G.C. (1988). Prevention and treatment of malaria. Lancet, i, 32-37.
- Eggelte, T.A., Gilis, H., Brabin, B. Draper, C. (1988) Demonstration of sulfadoxine and pyrimethamine in body fluids after intake of Fansidar. Proceedings XIIth International Congress for Tropical Medicine and Malaria, p. 37.
- Eslava, A., Morgan, M., Voller, A., Bidwell, D.E. (1988). ELISA - a possible alternative to establish a therapeutic drug monitoring system in severe and complicated falciparum malaria. Transactions of the Royal Society of Tropical Medicine and Hygiene. 82:683-683.
- Freier, C., Alberici, G.F., Turk, P., Baud, F. & Bohoun, C. (1986a). A radioimmunoassay for the antimalarial drug chloroquine. Clinical Chemistry, 32, 1742-1945.
- Freier, C., Alberici, G.F., Andriex, J. & Bohoun, C. (1986b). Monoclonal antibodies to lipophilic and short-sized haptens: application to the 4-amino-quinoline antimalarial drugs. Molecular Immunology, 23, 793-797.
- Greenwood, B.M., Greenwood, A.M., Bradley, A.K., Shenton, F.C., Smith A.C., Snow, R.W., Williams, K., Eggelte, T.A., Huikeshoven, H. & De Wit, M. (1986). ELISA tests for dapsone and pyrimethamine and their application in a malaria chemoprophylaxis programme. Bulletin of the World Health Organization, 64, 909-916.
- May, K. (1988). In: W.P. Collins, Home testing Complementary Immunoassays, John Wiley & Sons.
- Morgan M.R.A., Branham, S., Web, A.J., Robinas, R.J. & Rhones, M.J.C. (1985). Specific immunoassays for quinine and quinidine: comparison of radioimmunoassay and enzyme-linked immunosorbent assay procedures. Planta Medica, 3, 237-241.
- Onori, E. (1984). The problem of Plasmodium falciparum resistance in Africa South of the Sahara. Bulletin of the World Health Organization, 62, (supplement) 55-62.

Peters, W. (1987). Chemotherapy and drug resistance in malaria. Academic Press.

Rowell, V. & Rowell, F.J. (1987). A rapid enzyme-linked immunosorbent assay (ELISA) with a visual end-point for detecting quinine in urine, serum and dried blood. *Analyst*, 112, 1437-1439.

Shenton, F. Bots, M., Menon, A., Eggelte, T.A., de Wit M. & Greenwood, B.M. (1988). An ELISA for detection of chloroquine in urine. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 82, 216-220.

Sidki, A.M., Al-Abdulla, I.H. & Rowell, F.J. (1987a). Quinine directly determined in serum or urine by separation fluorimmunoassay. *Clinical Chemistry*, 33, 463-467.

Sidki, A.M., Al-Abdulla, I.H., Landon, J. & Rowell, F.J. (1987b). Polarization fluorimmunoassay for quinine in serum and urine. *Southeast Asian Journal of Tropical and Medical Public Health*, 18, 149-155.

Spencer, H.C. (1985). Drug-resistant malaria-changing patterns mean difficult decisions. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 79, 748-758.

Van der Kaay, Eggelte, T.A., Wetsteyn, J., Mutabingwa, M. (1988). Use of an ELISA test for the detection of chloroquine in body fluids. *Proceedings XIIth International Congress for Tropical Medicine and Malaria* (1988) pp. 352.

Ward, C.M. & Morgan, M.R. (1988). An immunoassay for the determination of quinine in soft drinks. *Food Additives and Contaminants*, 5, 555-561.

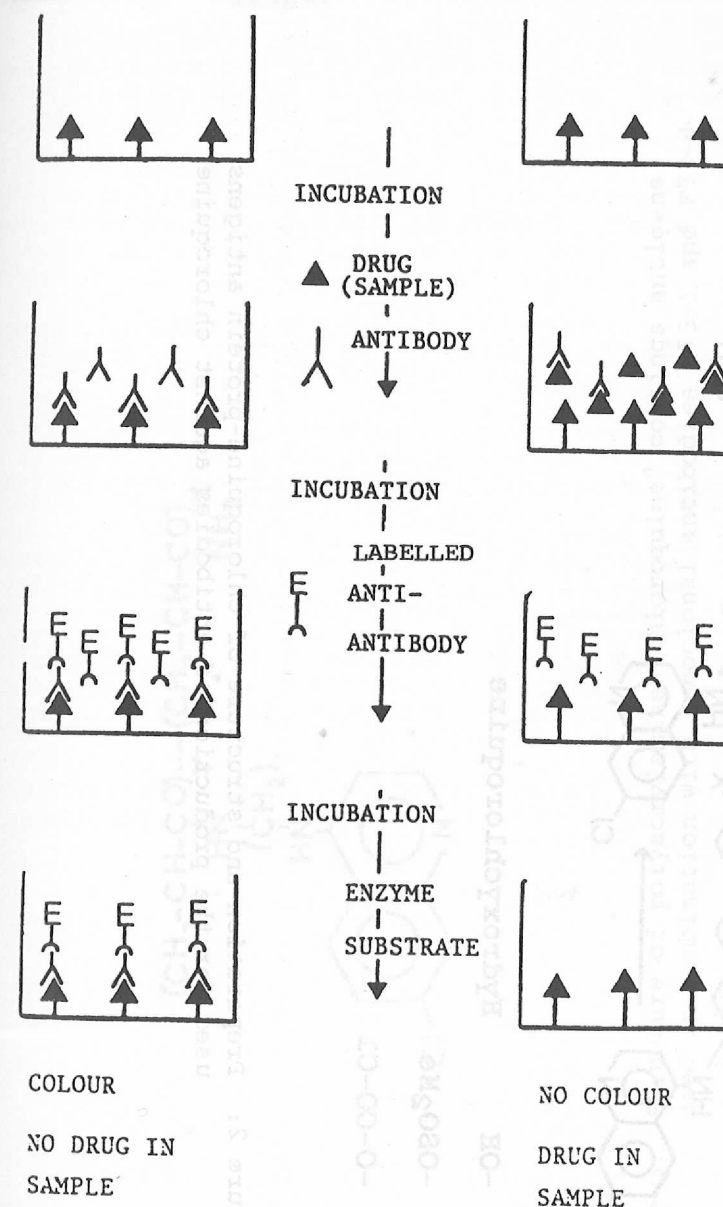


Figure 1: Indirect ELISA inhibition test: competition between solid phase bound drug and drug in solution for binding with drug specific antibody. Antibody binding is detected using an enzyme labelled second antibody.

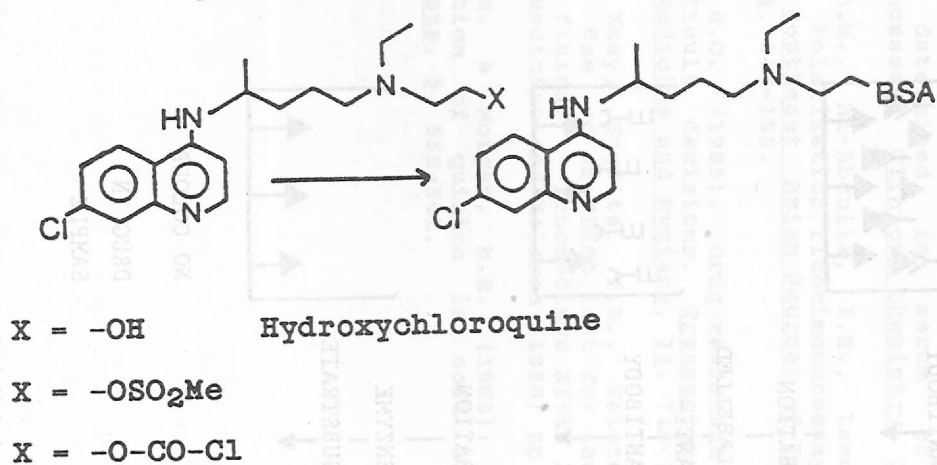


Figure 2: Preparation and structure of chloroquine-protein antigens used for the production of antibodies against chloroquine

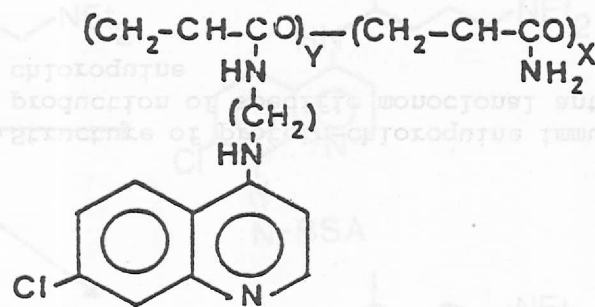


Figure 3: Structure of polyacrylamide-"chloroquine" coatings antigens used in combination with monoclonal antibodies F73-1 and F73-8

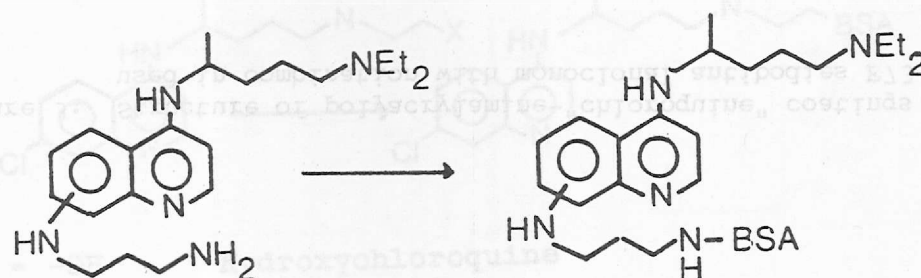


Figure 4: Structure of protein-chloroquine immunogen used for the production of specific monoclonal antibodies against chloroquine

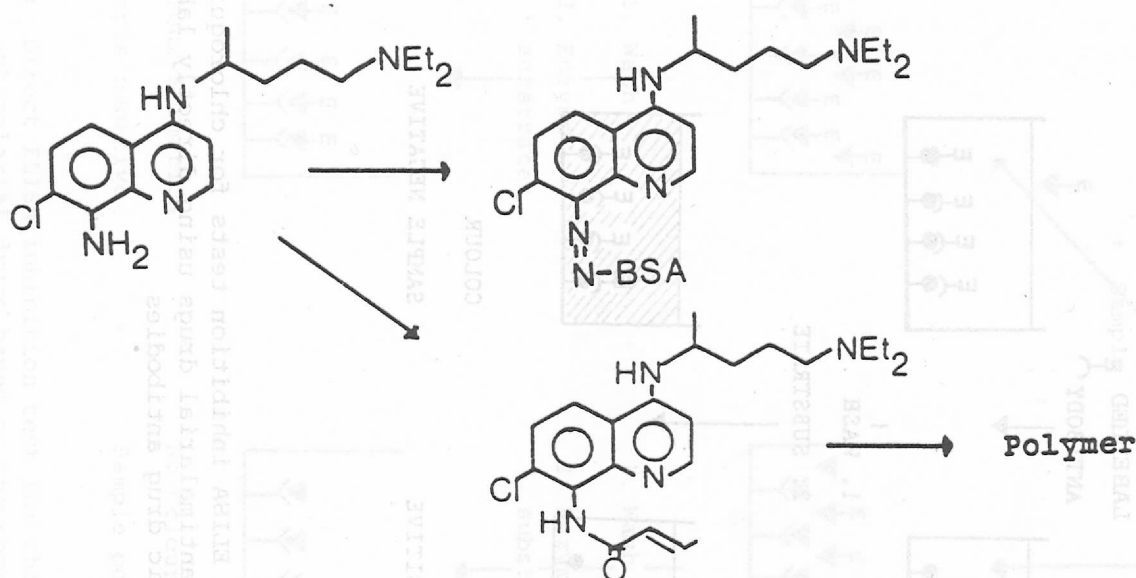


Figure 5: Structure of chloroquine antigens prepared from 8-aminochloroquine

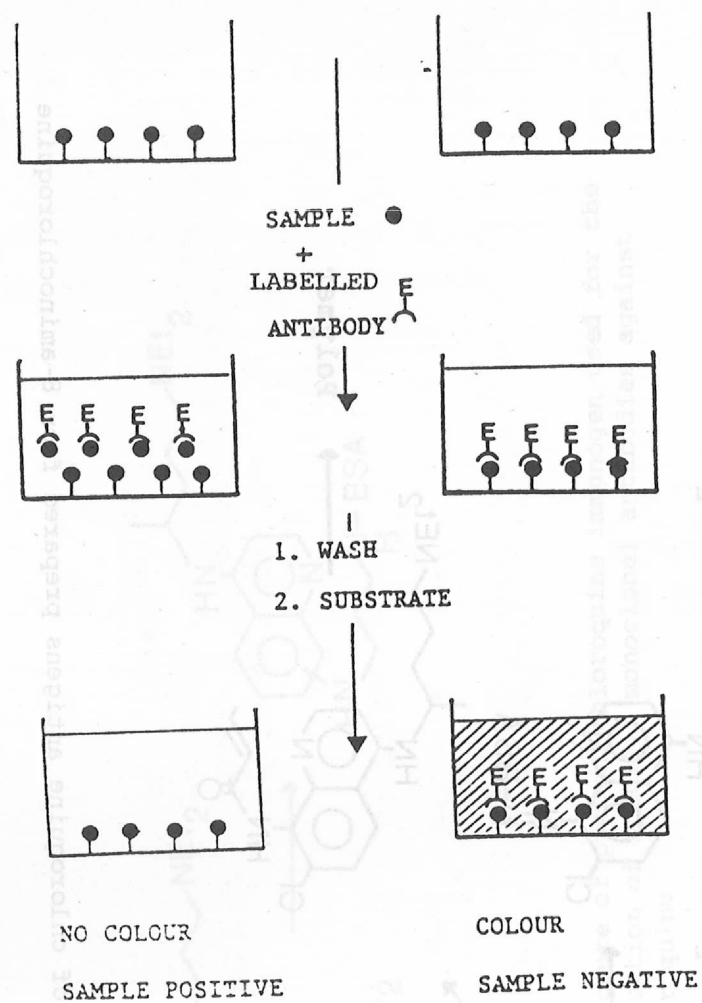


Figure 6: Direct ELISA inhibition tests for chloroquine and other antimalarial drugs using directly labelled specific drug antibodies

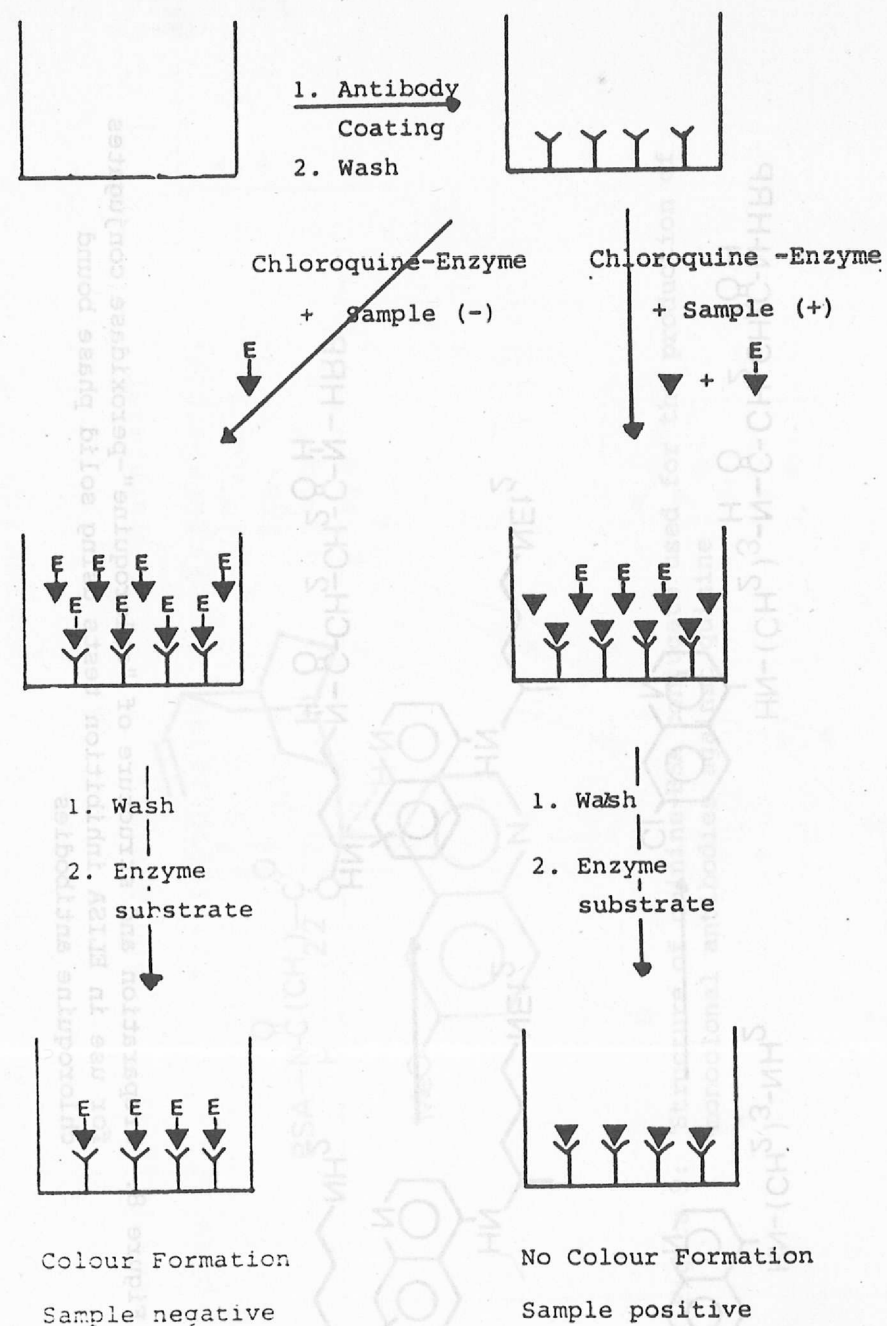


Figure 7: Direct ELISA inhibition test for chloroquine and other antimalarial drugs based on the competition of drug and enzyme labelled drug for binding with solid phase bound specific drug antibody

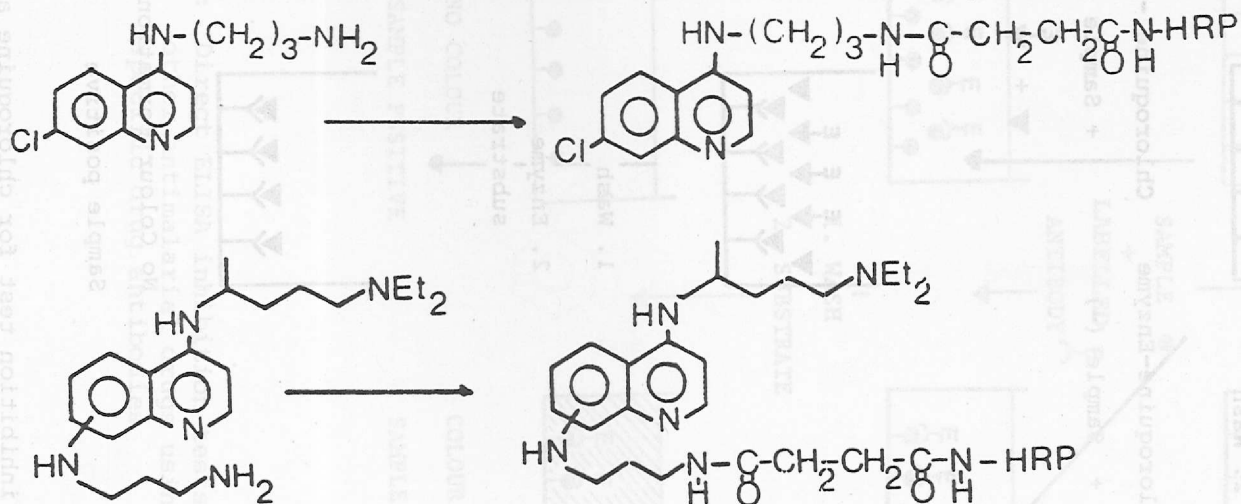


Figure 8: Preparation and structure of "chloroquine"-peroxidase conjugates for use in ELISA inhibition tests using solid phase bound chloroquine antibodies

Figure 9: Structure of quinine-BSA conjugate used for the production of monoclonal antibodies against quinine

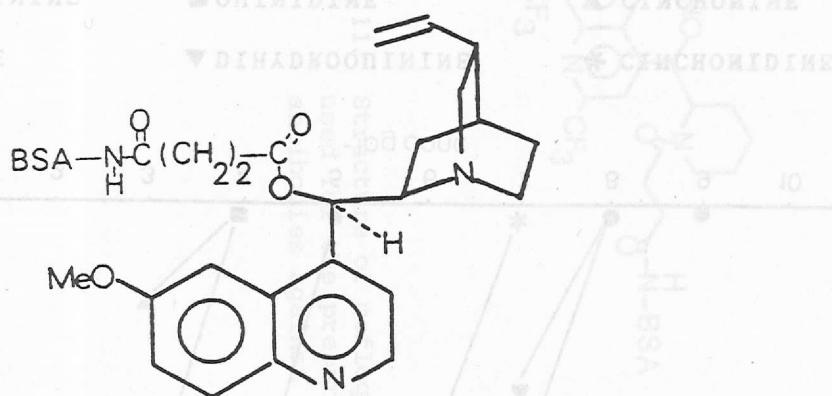


Figure 9: Structure of quinine-BSA conjugate used for the production of monoclonal antibodies against quinine

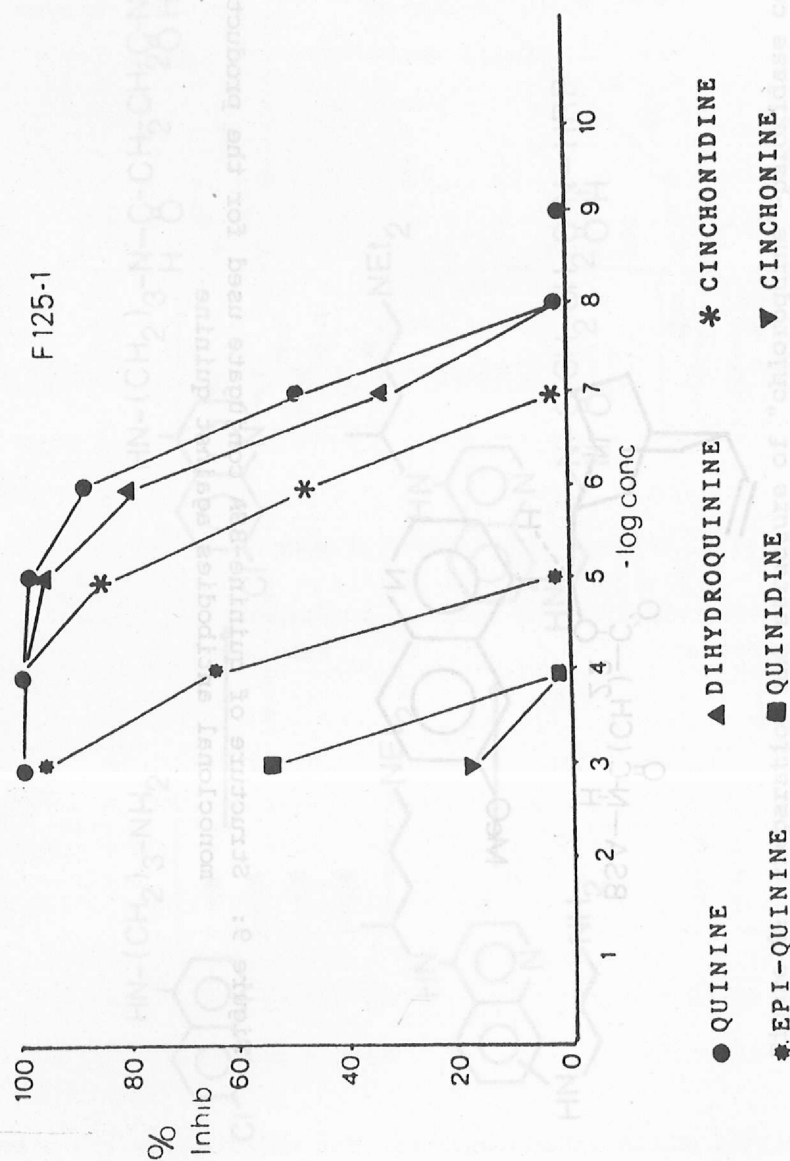


Figure 10: Cross-reactivity of cinchona alkaloids with quinone monoclonal antibody F125-1 used for ELISA inhibition test of quinone

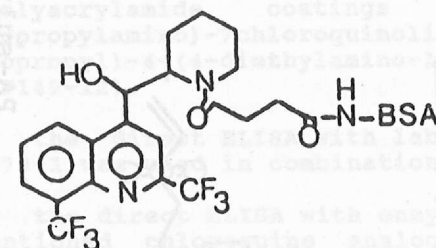


Figure 11: Structure of mefloquine-BSA conjugate used for the preparation of monoclonal antibodies against mefloquine

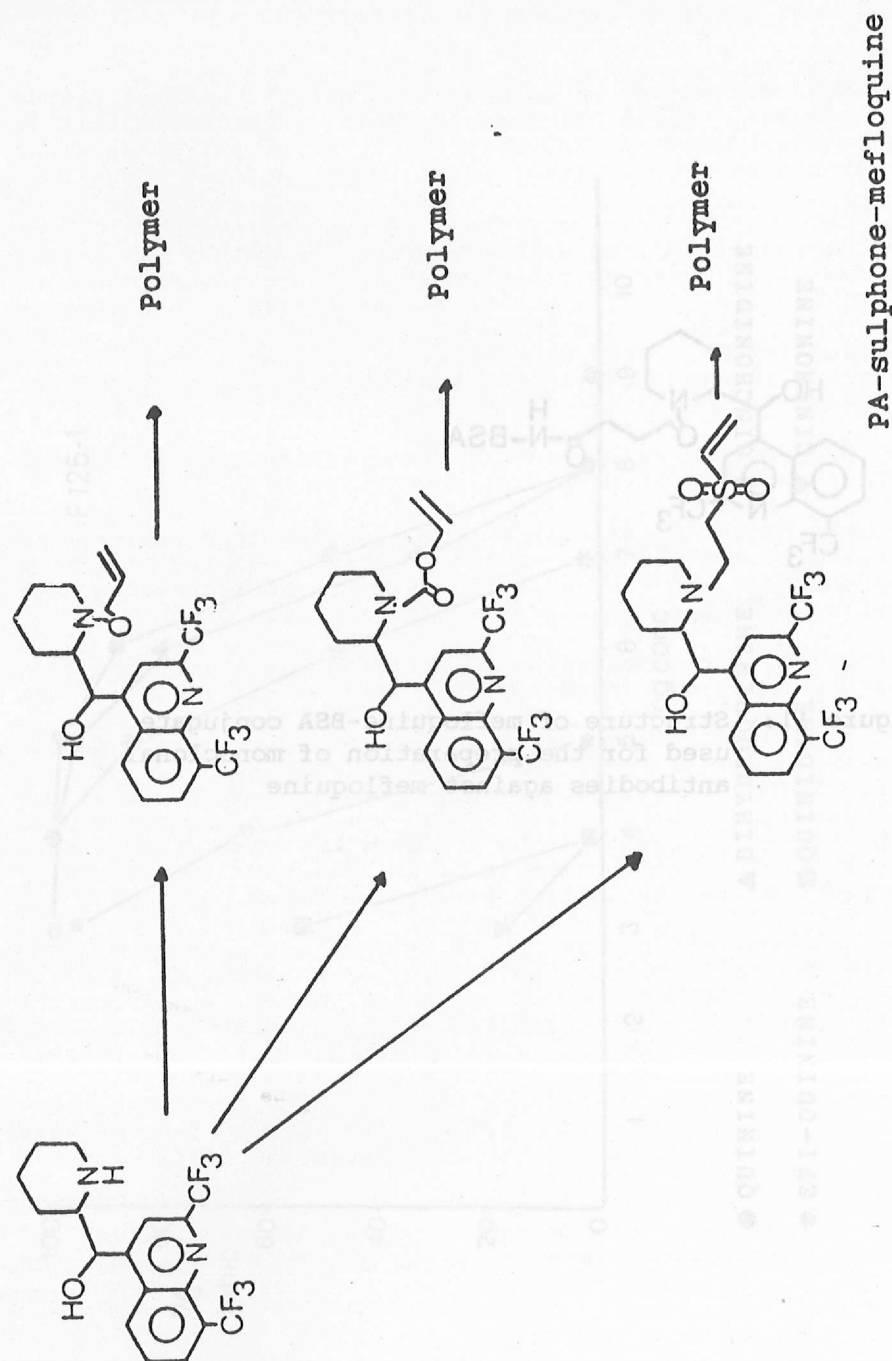


Figure 12: Preparation and structure of polyacrylamide-mefloquine coatings antigens

DETECTION OF CHLOROQUINE, QUININE AND MEFLOROQUINE IN URINE USING ELISA INHIBITION

For the detection of chloroquine, quinine and mefloquine in urine samples, three different types of ELISA inhibition tests have been used: A. Indirect ELISA, B. Direct ELISA using labelled antibodies and C. Direct ELISA using labelled drugs

For chloroquine two different types of antibodies have been used, which are either specific for chloroquine, (F147-12), or cross reacting with chloroquine metabolites and amodiaquine (F73-1 and F73-8). These antibodies were used in combination with polyacrylamide coatings antigens derived from 7-(3-aminopropylamino)-7chloroquinoline, (PA-CQ13, F73-8), and 7-(3-aminopropyl)-4-(4-diethylamino-1-methylbutylamino)quinoline, (PA-CQ7, F149-12).

For the direct ELISA with labelled antibodies, peroxidase labeled F73-1 was used in combination with PA-CQ13.

For the direct ELISA with enzyme labelled chloroquine, the above mentioned chloroquine analogues were coupled to horse radish peroxidase and these chloroquine-enzyme conjugates used in combination with monoclonals F73-8 and F149-12.

Unlabelled and peroxidase labelled, quinine antibody F125-1 were used in combination with a BSA-quinine in the direct ELISA. In the direct ELISA with enzyme labelled quinine, a conjugate of 9-hemisuccinyl horse radish peroxidase was employed in combination with solid phase bound F125-1. For the indirect and direct ELISA for mefloquine, unlabelled and peroxidase labelled monoclonal antibody, F150-9 was used in combination with a PA-sulphone-mefloquine coating antigen. For the direct tests with enzyme labelled mefloquine, a peroxidase succinylmefloquine conjugate was used in combination with solid phase bound F150-9.

The concentrations of solid phase coatings antigens, coating antibodies, and the dilutions of antibodies, labelled antibodies, or labelled drugs, can be varied according to the sensitivity required. Urine samples were tested in 4 dilutions: 1:10, 1:100, 1:1000 and 1:10000. Results can be assessed visually, or the colour formation measured using a microtitreplate reader. Results can be compared with those obtained with spiked urines, by testing these in the same dilutions as the test samples and/or compared with a calibration series of the drugs.

Using critical conditions, urine concentrations of chloroquine, mefloquine and quinine of 5-25 ug/L may be detected.

General Procedures ELISA Inhibition Tests

A. Indirect ELISA

1. Add to each well of the the microtitre plate 100 ul of coating antigen, (5-250 ug/L), in 0.05 M carbonate buffer, (pH = 9.6)
2. Cover the plate and incubate for two hours at 37°C or coat overnight at room temperature.
3. Empty the plate and wash with two quick rinses with PBS/0.05% Tween-20, (PBS/Tween), and two one minute incubations with the washing buffer, (PBS/Tween).
4. Add to the wells of the microtitre plate 50 ul of the test samples diluted in PBS, and 50 ul of the drug calibration series.
5. Add 50 ul of an antibody dilution in PBS/0.05% Tween/2% BSA.
6. Mix the contents of the plate and incubate for one hour at 37°C.
7. Empty the plate and wash with PBS/Tween, (see 3 above).
8. Add 100 ul of a peroxidase anti-mouse conjugate dilution (PBS/0.05%Tween/1% BSA), to the wells of the plate.
9. Incubate for one hour at 37°C.
10. Wash the plate with PBS/Tween, (see 3 above).
11. Add to each well of the plate 100 ul of O.P.D. substrate solution.
12. After 15-20 minutes add 50 ul 1M. H₂SO₄ to each well of the plate.
13. Read the plate at 492 nm.

B. Direct ELISA Test: (Directly Labelled Specific Antibodies)

1. Add to each well of the microtitre plate 100 ul of coating antigen, (5-250 ng/ml), in 0.05 M carbonate buffer, (pH = 9.6)
2. Cover the plate and incubate 2 hours at 37°C or overnight at room temperature.
3. Empty the plate and wash with two quick rinses with PBS/0.05% Tween, (PBS Tween), and two one minute incubations with the washing buffer, (PBS/Tween).
4. Add to the wells of the microtitre plate 50 ul of the test samples diluted in PBS, and 50 ul of the drug calibration series.
5. Add 50 ul of a labelled antibody dilution in PBS/0.05% Tween/2% BSA
6. Mix the contents of the plate and incubate for one hour at 37°C.
7. Empty the plate and wash with PBS/Tween, (see 3 above).
8. Add to each well of the plate 100 ul of O.P.D. substrate solution.
9. After 15-20 minutes add 50 ul 1M. H₂SO₄ to each well of the plate.
10. Read the plate at 492 nm.

C. Direct Test: (Enzyme Labelled Drug)

1. Add to each well of the microtitre plate 100 ul of a dilution of the specific monoclonal antibody, (2-10 mg/L), in PBS.
2. Cover the plate and incubate overnight at room temperature.
3. Empty the plate and wash with two quick rinses with pBS/0.05% Tween-20, (PBS/Tween), and two one minute incubations with the washing buffer, (PBS/Tween).
4. Add to wells of the microtitre plate 50 ul of the test samples diluted in PBS, and 50 ul of the drug calibration series.
5. Add 50 ul of an enzyme labelled drug dilution in PBS/0.05% Tween/2% BSA.

6. Mix the contents of the plate and incubate for one hour at 37°C.
7. Empty the plate and wash with PBS/Tween, (see 3 above).
8. Add to each well of the plate 100 ul of O.P.D. substrate solution.
9. Add after 15-20 minutes 50 ul 1M. H₂SO₄ to each well of the plate
10. Read the plate at 492 nm.

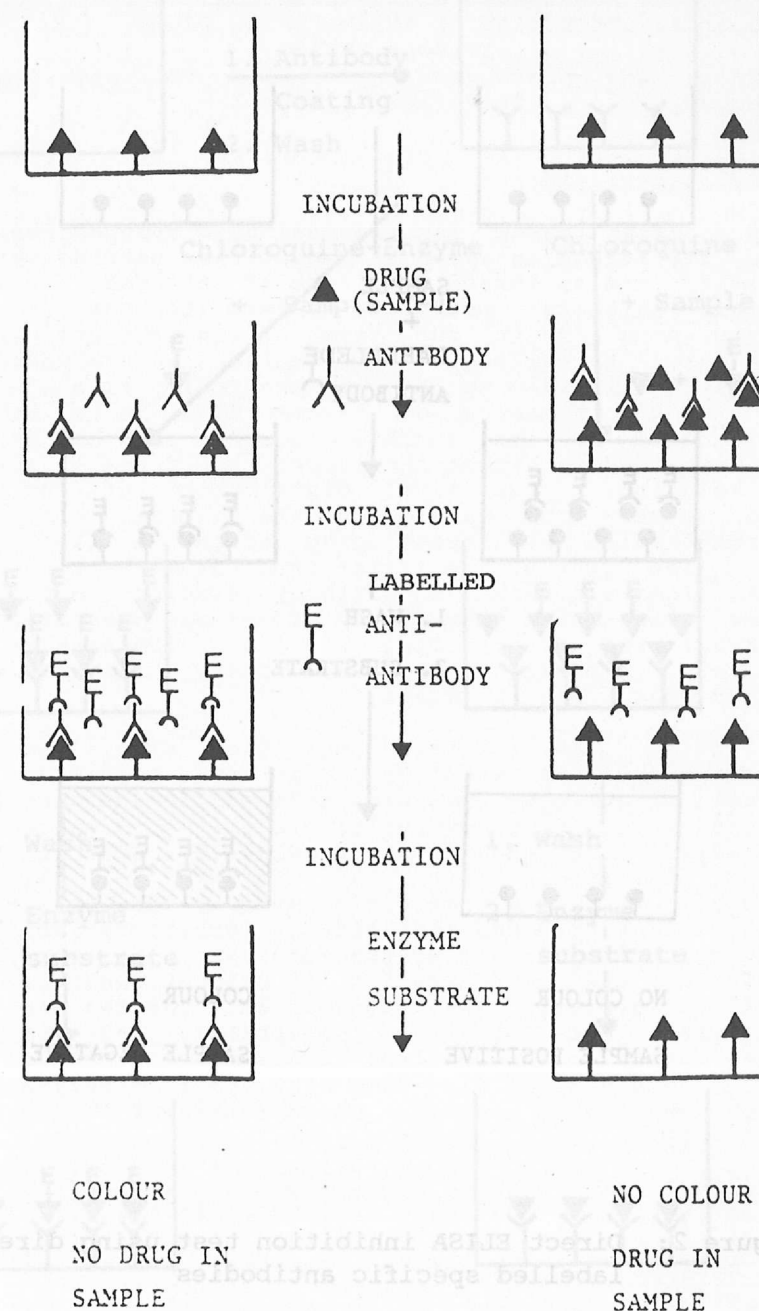


Figure 1: Indirect ELISA inhibition test

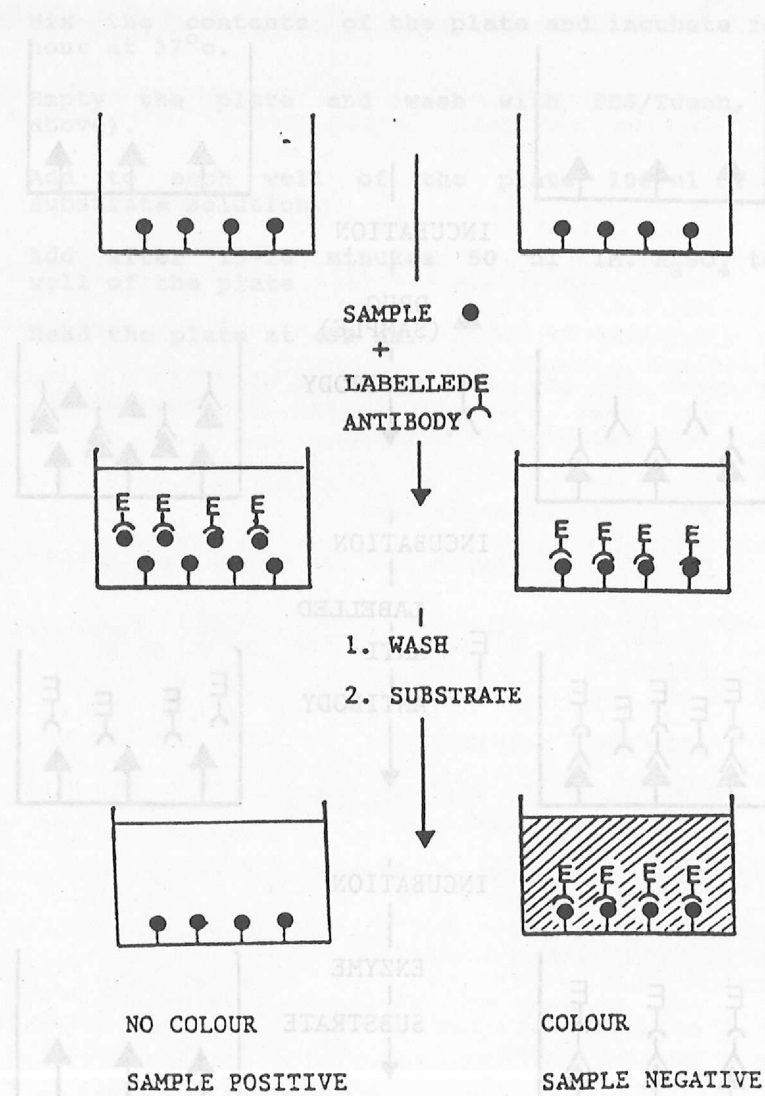


Figure 2: Direct ELISA inhibition test using directly labelled specific antibodies

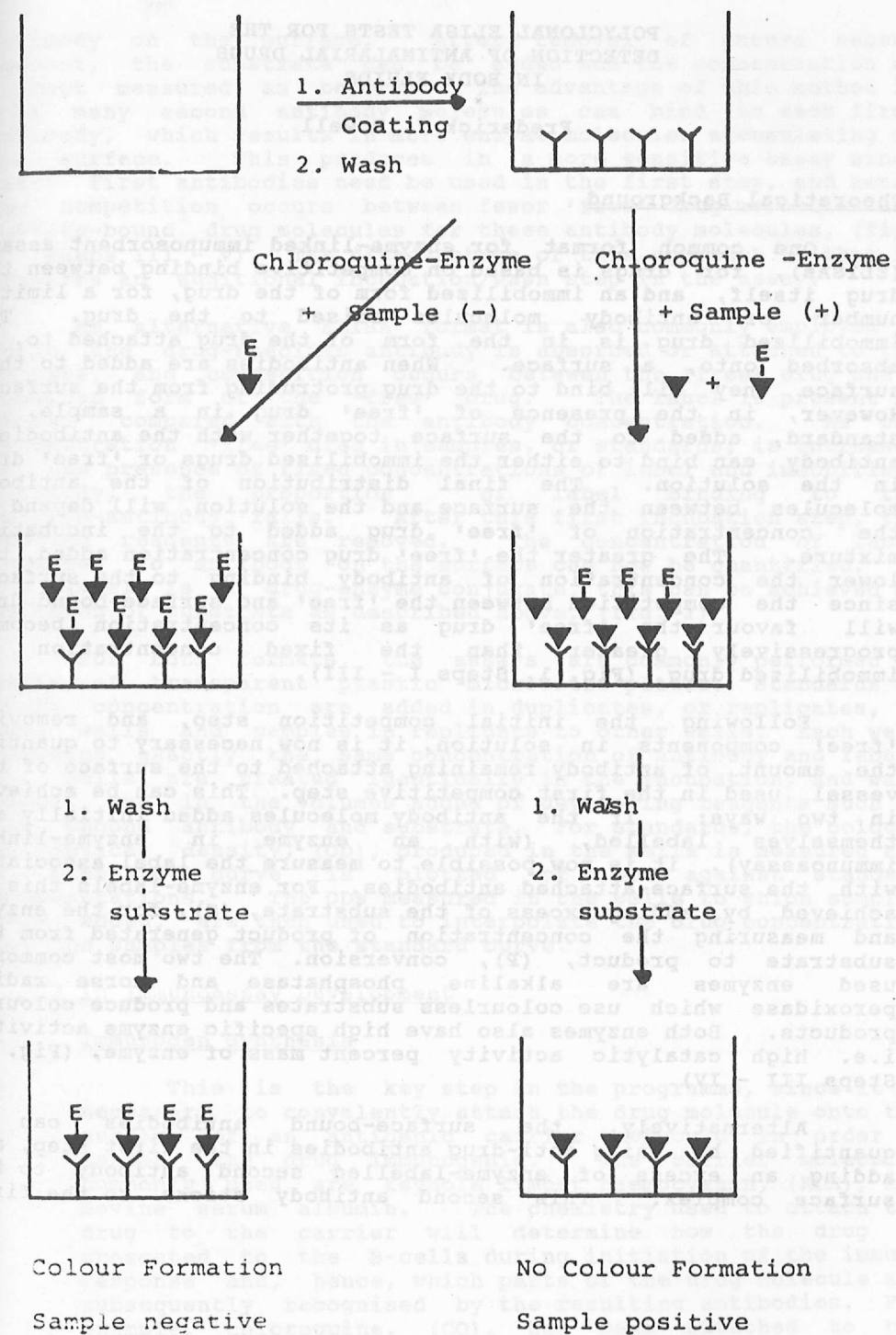


Figure 3: Direct ELISA test using enzyme labelled drugs

POLYCLONAL ELISA TESTS FOR THE DETECTION OF ANTIMALARIAL DRUGS IN BODY FLUIDS

Frederick J. Rowell

Theoretical Background

One common format for enzyme-linked immunosorbent assays (ELISAs), for drugs is based on competitive binding between the drug itself, and an immobilized form of the drug, for a limited number of antibody molecules raised to the drug. The immobilized drug is in the form of the drug attached to, or absorbed onto, a surface. When antibodies are added to this surface they will bind to the drug protruding from the surface. However, in the presence of 'free' drug in a sample, or standard, added to the surface together with the antibodies, antibody can bind to either the immobilized drugs or 'free' drug in the solution. The final distribution of the antibody molecules between the surface and the solution, will depend on the concentration of 'free' drug added to the incubating mixture. The greater the 'free' drug concentration added, the lower the concentration of antibody binding to the surface, since the competition between the 'free' and surface-bound drug will favour the 'free' drug as its concentration becomes progressively greater than the fixed concentration of immobilized drug, (Fig. 1, Steps I - III).

Following the initial competition step, and removing 'free' components in solution, it is now necessary to quantify the amount of antibody remaining attached to the surface of the vessel used in the first competitive step. This can be achieved in two ways: If the antibody molecules added initially are themselves labelled, (with an enzyme in enzyme-linked immunoassay), it is now possible to measure the label associated with the surface-attached antibodies. For enzyme-labels this is achieved by adding excess of the substrate, (S), for the enzyme and measuring the concentration of product generated from the substrate to product, (P), conversion. The two most commonly used enzymes are alkaline phosphatase and horse radish peroxidase which use colourless substrates and produce coloured products. Both enzymes also have high specific enzyme activity, i.e. high catalytic activity percent mass of enzyme, (Fig. 1, Steps III - IV).

Alternatively, the surface-bound antibodies can be quantified by using anti-drug antibodies in the first step, and adding an excess of enzyme-labelled second antibody to the surface complex. This second antibody sticks to the first

antibody on the surface. After removal of excess second reagent, the substrate can be added and the concentration of product measured as before. The advantage of this method is that many second antibody molecules can bind to each first antibody, which results in more enzyme molecules accumulating on the surface. This produces in a more sensitive assay since fewer first antibodies need be used in the first step, and hence the competition occurs between fewer 'free' drug molecules and surface-bound drug molecules for these antibody molecules, (fig. 1, steps III - V). The disadvantage of this approach is that it requires an additional incubation/wash step in the assay.

An alternative ELISA format is also commonly employed in which the drug-specific antibody is absorbed or attached to the surface, and competition occurs between the 'free' drug and a labelled form of the 'free' drug. The label is present in excess compared with the antibody concentration. As the concentration 'free' drug in samples, or standards, is increased in the presence of fixed concentrations of label and immobilized antibody, the proportion of label binding to the surface-antibody falls. After this first incubation step, the unbound reagents are removed. The concentration of label attached to antibody on the surface can now be quantified. If the label is a drug-enzyme conjugate, this can be achieved by addition of substrate as described above, (fig. 2)

For both formats, the assays are commonly performed in wells of transparent plastic microtitre plates. Standards of known concentration are added in duplicates, or replicates, to some wells and samples in replicate to other wells. Each well contains exactly the same concentration of antibody and label. All wells are treated identically in the incubation, and wash steps, and in the volumes added of developing reagents such as the second antibody and substrate. For standards, the colour, or optical density, (OD), produced in the wells is measured and a standard curve is plotted of OD against standard concentrations. The ODs measured in the wells to which samples were added, are then used to interpolate the drug concentration for the samples from the standard curve.

Steps in Immunoassay Development

1. Immunogen Synthesis

This is the key step in the programme, since it is necessary to covalently attach the drug molecule onto the surface of an antigenic carrier molecule in order to render the drug immunogenic. The carrier molecules commonly used are keyhole limpet haemocyanin, (KLH), and bovine serum albumin. The chemistry used to attach the drug to the carrier will determine how the drug is presented to the B-cells during initiation of the immune response and, hence, which parts of the drug molecule are subsequently recognised by the resulting antibodies. For example, chloroquine, (CQ), has been attached to the

carrier protein KLH through its side chain, (via the didesethyl CQ metabolite), or through an aminoethyl bridge substituted into position 6 or 9 of quinoline nucleus. Antisera raised in sheep to the former immunogen nucleus cross-react equally with CQ and its mono- and didesethyl metabolites whereas sheep antisera, raised to the latter immunogen are specific for the parent drug. Therefore, it is necessary to consider the required specificity of the assay, and the uses to which the assay will be put, when designing the immunogen.

2. Immunization

High titre antisera have been produced to antimalarial drugs in sheep and rabbits. My group have used sheep to generate large volumes of antisera to chloroquine, (two immunogens), quinine, and primaquine (Rowell et al., 1988; Rowell & Rowell, 1987; Al-Abdulla, (1987). Other workers have used rabbit, e.g. Ravindran et al., (1988) for primaquine and Morgan et al., (1985) for quinine.

3. Testing for Antibody

If a radioactive form of the drug is available, then this may be used in a conventional radioimmunoassay format to assess for the presence of antibody, (Morgan et al., 1985). Failing that, a fluorescent derivative may be prepared and the serum screened for antibody by polarization fluoroimmunoassay. We have used this method of screening for antibody to quinine, (Sidki et al., 1987), and chloroquine, (Rowell et al., 1988). Alternatively, antibody can be screened using drugs immobilized onto the wells of microtitre plates, and enzyme-labelled second antibody as described above for the first ELISA format. This approach requires the synthesis of a drug-protein conjugate attachable by passive adsorption onto the microtitre plate wells, which is immunoreactive to the drug moiety only, and producing an antibody binding which is displaceable in the presence of the drug-analyte.

4. Assay Validation

Having demonstrated high titre antisera, and synthesized a coating-conjugate having appropriate anti-body binding properties, it is necessary to optimize the assay in order to obtain an assay of the required sensitivity, recovery, precision, sample-handling capacity and speed. Such optimization will partly be dependent on the expected concentration range in the biological fluid which is to be analysed. We have aimed to complete the assay, using a 96 well plate with standards and samples in triplicate, within 90 - 120 minutes. Our assays employ the immobilized drug and the enzyme-labelled second

antibody format. Precision is generally less than a 10% coefficient of variation for within, and between, assay replicates. Analytical recoveries are within the range 95 - 105%. The concentrations of coating conjugates, and first and second antibody and incubation times, are chosen to generate standard curves for the drugs covering the expected concentration range, whilst using the maximum amount of biological fluid in the initial incubation step. This volume of biological fluid, (0.5 - 5 ul of serum or urine), does not produce a matrix effecting the assay.

The final step in the usual validation process is to compare results obtained by the ELISA method with those obtained for the same samples by an established method. The results should be identical if the ELISA is performing correctly.

For ELISA to be used in the field, additional laboratory-based validation should be performed to test the stability of the reagents under conditions of elevated temperature, humidity and bright light. For the chloroquine ELISA we have exposed: coated plates sealed with foil; freeze-dried antisera in sealed bottles under nitrogen; enzyme-labelled second antibody in solution and chloroquine standards; and enzyme substrate, (both freeze dried in buffer in sealed bottles under nitrogen), to conditions of 100% humidity and 37°C for extended periods, (up to 48 hours). All reagents are stable under these conditions. However on reconstitution with solutions, the enzyme substrate para-nitrophenol phosphate, and the chloroquine standards deteriorate rapidly. Accordingly they should be used immediately after reconstitution.

Applications

ELISAs have the advantages of great sensitivity which allows direct analysis of biological samples without the need to extract the drug from the matrix. These assays also have the potential of great specificity if there are no compounds of similar structure present in the sample to cross-react in the assay. As noted, good design of the immunogen should preclude cross-reaction of structurally similar drug metabolites if an assay of great specificity is required.

The assay can be used in a fully quantitative manner if it shows negligible cross-reactivity with structurally similar drugs, and metabolites, and the precision and recovery of the assay are good. Potentially, a single plate of 96 wells can be used to analyse 26 samples, 6 standards and 2 quality control samples, in triplicate with 1 - 2 hours total assay time. Four to five such assays could be performed within one day, resulting in the analysis of over one hundred samples per day.

We have used the specific ELISA for CQ in this way to determine pharmacokinetic parameters for drug elimination in urine and whole capillary blood in the form of dried blood spots on filter paper, (Rowell et al., 1988). The assay can also be used in a qualitative manner to give an indication of the presence, or absence, of a drug in a sample of serum, urine, dried blood spot, etc. In this mode, a positive and negative standard are used in the assay, together with samples, and the colour produced in the wells of the samples at the end of the assay is compared to that of the standards. This visual endpoint can be used to discriminate the presence of relatively low concentrations of drug in the sample. For quinine, for example, the limit of visual detection is 30 ug/L for urine, and dried blood spots, and 100 ug/L for serum samples, (Rowell & Rowell, 1987).

Costs

The major cost in developing the assay is that associated with the production of high titre antisera. For sheep, it may take up to 6 months to acquire over one litre quantities of such antisera, and a whole assay may take up to 12 months to be fully established. However, once the antiserum is produced, 2 litres will be sufficient to enable the assay of 1 million 96-well microtitre plates. The cost of the ELISA for materials is about US\$ 1.50 per kit (26 samples in triplicate or 40 samples in duplicate), for the production of 1000 kits, which rises to about US\$40 per kit when labour costs, packaging and other costs are added.

Future Developments

The assay format described above still requires considerable pipetting skills. It involves three separate pipetting steps and two separate incubation steps, all of which are time-consuming, and requires a microtitre plate reader to provide quantitative results. Therefore, developments are needed which will simplify the method, whilst retaining the sensitivity and specificity inherent in immunoassays.

These disadvantages of the ELISA method have been addressed in recent developments of immunologically-based biosensors and dipsticks. An example of a simple biosensor, which has been applied to the measurement of large and small molecules, is the optical capillary-fill device. This device consists of two small plates of glass, 30 mm by 15 mm, separated by a small capillary gap, (100 μ m). All the reagents required for the assay are deposited on the inner surfaces of the plates, the immobilised antibody on the lower surface and fluorescently-labelled analyte on the facing surface.

A drop of sample, (serum, blood or urine), is applied to the open end of the capillary which fills to the same reproducible volume for each device. The sample dissolves the

tracer which is released into the space between the plates. Competition then ensues, between the tracer and analyte molecules, for the specific antibody molecules bound to the inner surface of the bottom plate. The amount of tracer eventually binding to this surface is inversely proportional to the amount of analyte present in the sample.

A pulse of light is directed at right angles to the plates, and the intensity of emitted radiation emerging close to the plane of the plate is measured. The bottom plate acts as an optical waveguide for the emitted radiation only when the tracer is bound to this surface via its specific antibody. Therefore, in the presence of analyte, little tracer binds to this surface and no signal emerges from the baseplate. Therefore, this method involves no pipetting, or separation steps, and provides a signal in a matter of minutes. A simple flashlamp pump light, (e.g. a cheap photographic flash), and optical detectors constitute the reader. The instrument is precalibrated with standards of the analyte and the samples then processed. Each sensor is used once then discarded. Such a device is based on the work of Bradley et al., (1987), and is undergoing commercial development for a variety of analytes including drugs, (although not antimalarial drugs), by Serono Diagnostics Ltd., U.K.

A dipstick or test-strip, analogous to pregnancy testing kits, could also be applied to the detection of anti-malarial drugs in biological fluids. In one such format, antibody reagent is immobilised on the surface of paper, or on a similar material. The drop of sample is added, followed by a drop of a second reagent. The specific anti-drug antibody is attached to the surface and the second reagent added at this stage is drug-labelled enzyme. If drug is present in the sample, it will bind to the specific antibody on the surface saturating the antibody binding sites. On adding a drop of the second reagent, no binding of the enzyme-reagent will ensue, and this reagent is removed from the surface by capillarity. Conversely, if no drug is present in the sample, antibody binding activity will be retained on the surface of the strip so that addition of the second reagent will result in drug-labelled enzyme molecules binding to their specific antibody molecules on the surface. Addition of the substrate for the enzyme results in no colour developing if drug was present in the sample, but with colour developing if the drug was absent.

Both the tests described above are designed to test for a single drug and can be performed only once per sensor, or strip. Their cost is likely to be about US\$0.25 and 1.00 for capillary-fill sensor and test-strips respectively. It should be noted that currently neither of these test systems is available for anti-malarial drugs, although antibodies and drug-enzyme reagents are available which could be used to develop such tests.

References

- Al-Abdulla, I.H. (1987). Immunoassays for Quinine and Primaquine, M. Sc. Thesis, University of London.
- Bradley, R.A., Drake, R.A.I., Shanks, I.A., Smith, A.M., and Stephenson, P.R. (1987). Optical Biosensors for Immunoassays; The Fluorescence Capillary-Flow Device. *Philosophical Transactions of the Royal Society of London, Series B*, 316:143 - 160.
- Morgan, M.R.A., Bramham, S., Webb, A.J., Robbins, R.J. and Rhodes, M.J.S. (1985). Specific immunoassays for quinine and quinidine: comparison of radioimmunoassay and enzyme-linked immunoassay procedures. *Plants Medica*, 3; 237 - 241.
- Sidki, A.M., Al-Abdulla, I.H., Landon, J. and Rowell, F.J. (1987). Polarization fluoroimmunoassay for quinine in serum and urine. *South-east Asian Journal of Tropical Medicine and Public Health*, 18; 149 - 155.
- Ravindran, B., Satapathy, A.K. and Das, M.K. (1988). Production and characterization of rabbit antibodies to primaquine. *Medical Science Research*, 16; 161 - 162.
- Rowell, V., Rowell, F.J., Baker, A., Laurie, D., and Sidki, A.M. (1988). A specific ELISA method of determining chloroquine in urine or dried blood spot. *Bulletin of the World Health Organization*, 66; 211 - 217.
- Rowell, V. & Rowell, F.J. (1987). Rapid enzyme-linked immunosorbent assay (ELISA) with a visual end-point for detecting quinine in urine, serum and dried blood spots. *Analyst*, 112, 1437 - 1439.

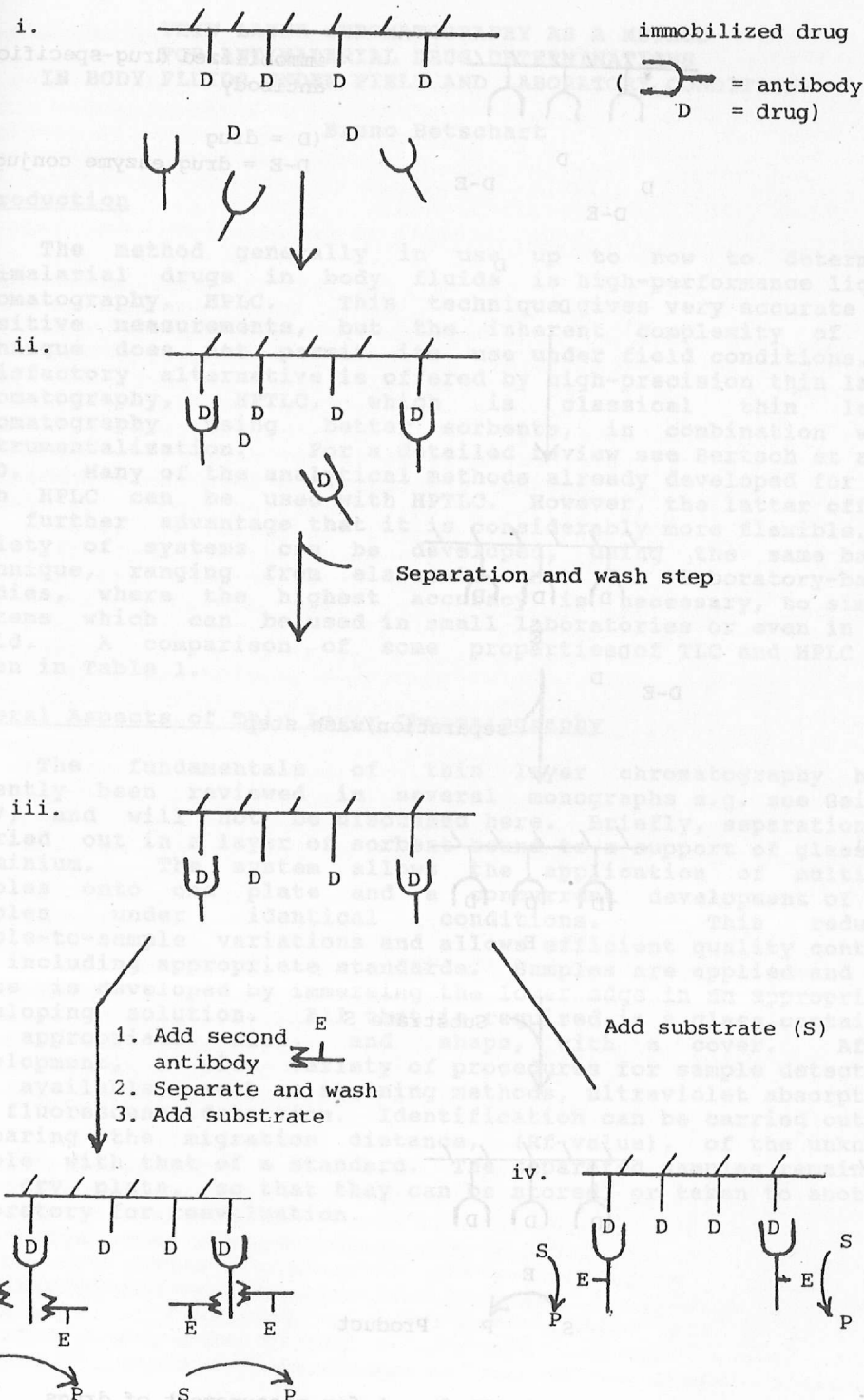


Figure 1: An ELISA for measurement of drugs (immobilized drug)

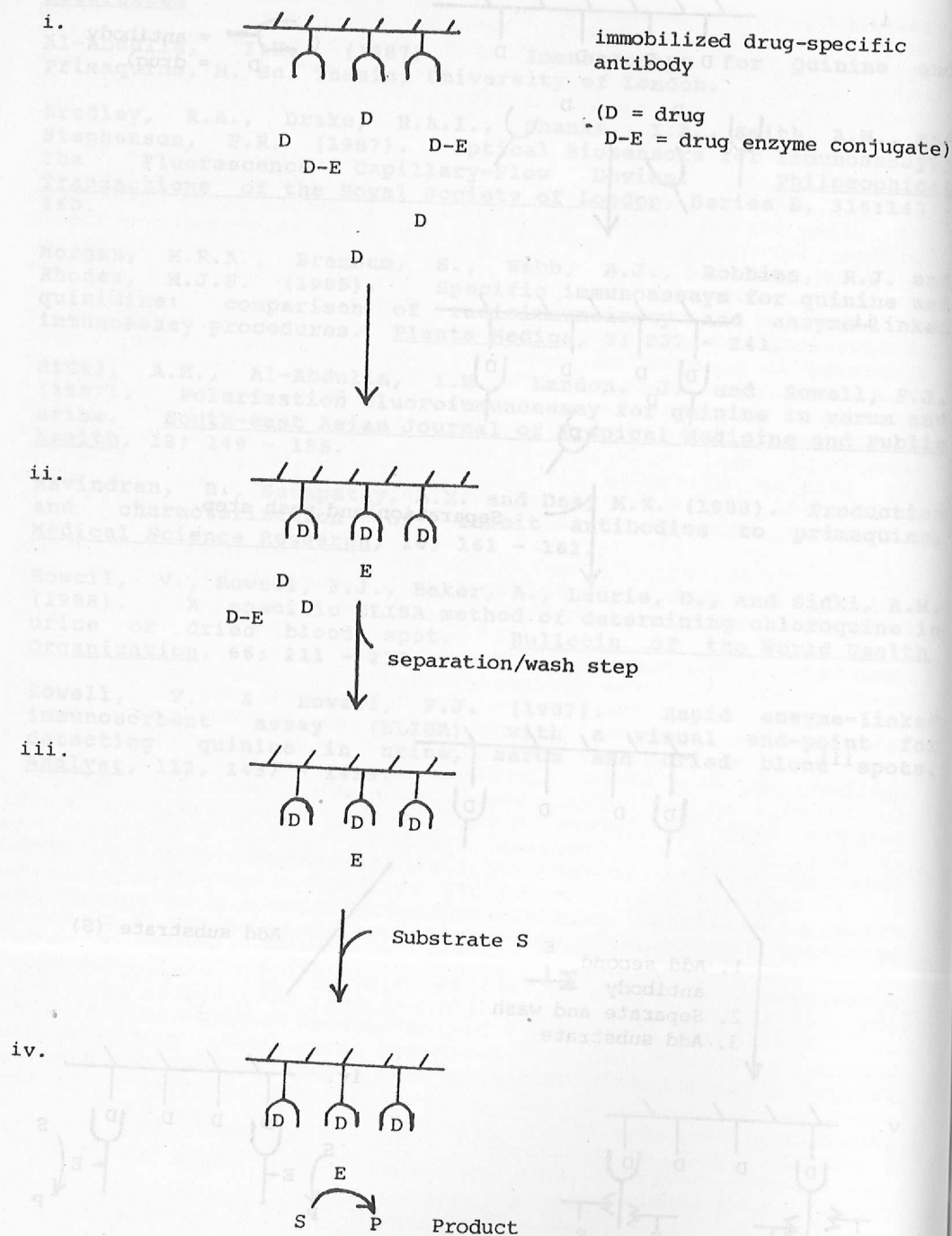


Figure 2: An alternative ELISA format for measurement of drugs (immobilized antibody)

THIN LAYER CHROMATOGRAPHY AS A METHOD FOR ANTIMALARIAL DRUG DETERMINATIONS IN BODY FLUIDS UNDER FIELD AND LABORATORY CONDITIONS

Bruno Betschart

Introduction

The method generally in use up to now to determine antimalarial drugs in body fluids is high-performance liquid chromatography, HPLC. This technique gives very accurate and sensitive measurements, but the inherent complexity of the technique does not permit its use under field conditions. A satisfactory alternative is offered by high-precision thin layer chromatography, HPTLC, which is classical thin layer chromatography using better sorbents, in combination with instrumentalization. For a detailed review see Bertsch et al., 1980. Many of the analytical methods already developed for use with HPLC can be used with HPTLC. However, the latter offers the further advantage that it is considerably more flexible. A variety of systems can be developed, using the same basic technique, ranging from elaborate ones for laboratory-based studies, where the highest accuracy is necessary, to simple systems which can be used in small laboratories or even in the field. A comparison of some properties of TLC and HPLC are given in Table 1.

General Aspects of Thin Layer Chromatography

The fundamentals of thin layer chromatography have recently been reviewed in several monographs e.g. see Geiss, 1987, and will not be discussed here. Briefly, separation is carried out in a layer of sorbent bound to a support of glass or aluminium. The system allows the application of multiple samples onto one plate and a concurrent development of all samples under identical conditions. This reduces sample-to-sample variations and allows efficient quality control by including appropriate standards. Samples are applied and the plate is developed by immersing the lower edge in an appropriate developing solution. All that is required is a glass container of appropriate size, and shape, with a cover. After development, a wide variety of procedures for sample detection are available, such as staining methods, ultraviolet absorption or fluorescence detection. Identification can be carried out by comparing the migration distance, (R_f-value), of the unknown sample with that of a standard. The separated samples remain on the dry plate, so that they can be stored, or taken to another laboratory for reevaluation.

There is a great variety of sorbents and developing solutions, so separation methods for a wide variety of substances can be developed. It is also possible to vary the conditions of development, e.g. vapour saturation. Linear development chambers can be used in which the plate is developed from both edges. This is economical because more samples can be applied and the solvent volume needed is small.

Differences Between Classical TLC and HPTLC

A number of developments have made it possible for TLC to evolve into a method which can be used for high-precision quantitative assays, (see Table 2). With the introduction of high precision thin layer plates, with a small particle size (typically 7 μm), and a narrower range of particle size, it became possible to reduce the optimal development distance to 3 - 5 cm compared with the 10 - 15 cm on conventional TLC plates. This results in a 4 - 6 times faster separation time, and a smaller spot size. Because the sample is more concentrated, the smaller spot size allows a lower detection limit. In order to provide precise and reproducible quantitative results, the amount of sample applied must be carefully controlled. The ideal sample volume is in the 100 - 500 nanolitre range, and this can best be applied using instrumentalized application systems. Larger volumes can also be applied by using this instrument for a bandwise sample application with an accurately controlled delivery rate. Whereas classical TLC plates can be evaluated qualitatively by eye, or by scraping the spots off the plates for photometric quantification, the HPTLC plates have to be densitometrically evaluated.

Major fields of HPTLC applications include food chemistry, environmental analysis, clinical chemistry, quality control in pharmaceutical industry as well as drug screening in forensic analysis.

Field Tests to Measure Antimalarial Drugs Based on Thin Layer Chromatography

Two laboratories, (Mount et al., 1988 and Betschart et al., 1990 *Acta Tropica*, in press), have developed field methods to determine antimalarial drugs. Thin layer chromatographic field methods are simple to carry out and reliable in the analysis of the data, (Mount et al., 1987; Mount et al., 1988; Churchill, 1989).

Mount et al., (1987), extracted chloroquine (CQ) and desethyl-chloroquine (DCQ), from 5 ml urine, or 100 μl blood, by adding the body fluids to centrifuge tubes containing dry sodium phosphate, or 20% aqueous trisodium phosphate dodecahydrate, (pH = 12.5). MTBE, (= methyl tert.-butyl ether), is added as organic solvent. The extraction is carried out by shaking or vortexing the tubes. The tubes are then left to stand so that the layers separate clearly. The CQ and DCQ are then in the organic layer. For samples extracted from urine, 10 μl of the

0.8 ml organic layer was applied in 0.5 μl droplets directly to HPTLC-silica gel plates using a repeating dispenser. In the case of blood samples, the whole organic layer, i.e. 0.5 ml, was transferred into a cylindrical vial, and evaporated to dryness with a cool-air blower fitted to a manifold system adapted to accommodate several samples. The extract residue was reconstituted in 25 μl MTBE and the whole sample applied onto the silica gel plate. The chromatographic development was carried out in a linear development chamber, from both sides of the plate towards the centre, in 18% methanol+ 8% concentrated ammonia in MTBE for urine or in 4% n-butylamine in MTBE for extracts from blood samples. Standards, at appropriate dilutions were run on the same plates for evaluation and quality control.

The extracted drugs were visualized by using different methods, but always under short-wavelength, i.e. 254 nm, ultraviolet light. One possibility was to use plates which were impregnated with a fluorescence indicator. CQ and DCQ spots caused quenching of the fluorescence of the indicator, resulting in a dark spot. If plates without a fluorescence indicator were used, the CQ and DCQ spots showed a blue fluorescence. This method is more sensitive than the fluorescence quenching technique and gave a detection limit of 25 $\mu\text{g/L}$. The amount of the drugs was estimated semiquantitatively by visually comparing the intensity of the spots with those of standard samples, or by using a compact, battery-operated, spot luminance meter, (Churchill 1989). This method was shown to have a high specificity for CQ and DCQ and to allow a semiquantitative or quantitative evaluation and have a detection limit between that obtained with the chemical assays, e.g. 1 mg/L , and the ELISA, e.g. <25 $\mu\text{g/L}$. Where electrical power is available, the technique can be used in a moderately well-equipped laboratory to dry the samples. The extraction procedure is time consuming and it also involves careful work practices and laboratory-experienced personnel. This limits its wide application under field conditions.

The method to determine CQ in urine developed in our laboratory is still simpler and faster; two samples can be assayed in within 20 minutes. (See annex for the detailed description and Betschart et al., 1990, *Acta Tropica* in press). Three simple steps are needed to determine the presence or absence of CQ and its metabolites:

1. Urine samples are directly spotted in small droplets onto aluminium-backed HPTLC-silica plates, using, for example, a micropipette or a syringe. Although this is a field method, HPTLC-plates have to be used to guarantee the necessary sensitivity.
2. The plates are developed either in a simple beaker glass, or in a special chromatography device, with toluene: diethylamine: methanol in the ratio (8: 1: 1).

3. After chromatographic development, the plates are examined in complete darkness under UV light supplied by a solar-powered, hand-held lamp. The presence of the CQ-specific fluorescent band is indicative for the recent consumption of CQ. By including appropriate standards it is possible to identify the drug as well as to estimate its concentration in the urine.

The plates used have the sorbent bound on to aluminium-foil rather than glass. This offers many advantages. The plates are lighter, 0.5 kg compared with 3.6 kg. They are not easily broken, even under rough transport conditions, and they can easily be cut to an appropriate size to accommodate smaller, or larger, numbers of samples. If only a few, e.g. less than three samples, have to be analysed, a 5 X 10 cm plate can be used. A 20 X 10 cm plate can be used with a simple glass tank, for up to 12 samples. If a linear development chamber is available, so that plates can be developed from both sides, 24 samples per plate can be handled. If 4 standards are included then it is possible to assay 20 samples per plate.

If 25 μ l of urine is applied onto the plate, it is easily possible to detect 1 mg CQ-base per litre of urine. The application of the urine sample is simple, provided that some type of micropipetting device is available: This can be a micro-capillary or an Eppendorf-type pipette. The chromatographic properties of the silica gel are such that the antimalarial drugs are concentrated in a spot at the site of application. Almost no sideways diffusion i.e. chromatography, occurs under the polar conditions which result when the sample is applied dissolved in water, as is the case when urine is used directly. This phenomenon leads to an effective concentration of the antimalarial drugs. It is possible to spot the urine samples at a distance of 1.5 cm from each other; even though the urine spots spread, and may overlap, the drug remains concentrated. After air drying, which can be speeded by the use of a hair dryer, if one is available, the plates can be developed.

The solvents to develop the plates have been optimized to allow a sufficient separation of the antimalarials tested. Instead of the simple toluene: diethylamine mixture originally used for CQ, (Betschart & Steiger, 1986) a three component mixture is to be preferred, containing toluene, diethylamine and methanol. This mixture is stable for many weeks when stored in a tightly closed bottle. In the linear chamber, maximally 10 ml of solvent is needed to develop one 10 X 20 cm plate. The development is carried out under conditions in which the air is not saturated with solvent. This can be either in a glass container without a filter paper lining, or in the linear development chamber with the silica layer facing upwards. The separation is complete after 15 minutes. The alkaline pH of the developing solvent enhances the fluorescence of chloroquine and its metabolites. This allows an immediate reading of the plate after chromatographic development. The fluorescence is not

stable under these conditions, (Betschart & Steiger, 1986), which means that, if the results cannot be read within 20 minutes, sensitivity is reduced. However, if the plates cannot be read immediately after the run, or have to be reevaluated for quality control purposes later on, they can be bathed in a solution of 40% paraffin in hexane. This preserves the fluorescence for several weeks. It also offers the advantage that the results can be maintained as a record for quantitative, densitometric reevaluation in a centralized laboratory.

The specificity of the technique is very high owing to the combined selectivity of the chromatographic separation, and the specific fluorescence under broad range excitation, as provided by a simple type of UV light. The reproducibility of the determination is very good. Since the results depend on accurate application, and the observer's subjective estimation of the intensity of fluorescence, some variation is unavoidable. But it is within the limits set by the included standards.

To assess the predictive value of the field test, a controlled study was carried out in our laboratory at the Swiss Tropical Institute, by giving 5 volunteers a single dose of 300 mg and six other chloroquine base volunteers a single tablet of 100 mg chloroquine base. Daily urine samples were collected and analysed by applying directly either 20, or 50 μ l, aliquots onto the HPTLC-silica plates. The plates were evaluated by checking them visually for the presence of the CQ as well as the DCQ band. Densitometry was then carried out so that the visual results could be correlated with the quantitative values, (fig. 1). A single dose of 300 mg resulted in high values of CQ, (>6 mg/L) excreted in the first day after intake. After this, all values dropped though individual fluctuations were high. With a 20 μ l sample, it was possible to detect by eye concentrations of chloroquine base down to 0.8 mg/L. Chloroquine could be detected up to 10 days. When the volume of the urine sample was increased to 50 μ l, so that 0.4 mg base/L could be detected, it was possible to find CQ up to 36 days after drug consumption. Using densitometric evaluation, CQ could be detected up to 60 days after the single dose of 300 mg base. After a single dose of 100 mg base, and application of 50 μ l urine aliquots, samples with CQ could be detected by eye only up to day 5.

A surprising result was that, in both groups, the excretion did not decrease in an even fashion. There were strong individual daily fluctuations up to a factor of 5. This phenomenon needs to be studied in more detail before it can be established whether urine levels are reliable enough to be used as an indicator for the corresponding blood levels, (Mount et al., 1989). It is possible that the fluctuation in the CQ concentrations varies in the same individual on a daily basis due to differences in liquid consumption.

Applicability of (HP)TLC-Methods for the Determination of Antimalarial Drugs

It is possible to use thin layer chromatography in a modular way, building up a system with different components to make it appropriate for specific situations and purposes. A number of criteria have to be considered to permit the choice of the best system. The following are some questions which could be asked:

1. What is the objective of the study?

- Have the pre- and post-medication levels to be monitored?
- Is a study planned to relate the drug consumption in a population with resistance patterns?
- Is it necessary to control the quality of locally available tablets?

2. What kind of data are needed to reach these objectives?

- Have determinations to be carried out on the spot to obtain a quick answer about the presence of the drug?
- Is the sample size, which is to be analyzed, large or small?
- Are qualitative or semiquantitative data sufficient, or is it necessary to have quantitative results?
- Is it sufficient to determine drug concentrations in urine, or are determinations on whole blood, plasma or erythrocytes needed?

3. Which technical restraints exist?

- Has the determination to be made outside of a laboratory facility?
- Is there electrical power available?
- Is the study to be carried out by well-trained laboratory personnel?
- Has the test system to withstand rough roads and adverse environmental conditions, such as like high temperatures and high humidity?

4. What are the financial restraints?

The answers to these questions can provide guidelines for the selection of the optimal chromatographic system for the study. For example, if the objective is to study drug consumption in a population, the simple field test can be used anywhere to monitor, quickly, the consumption of specific antimalarial drugs in a large population, and requires only simple instructions and instrumentation. The response is simply 'Yes' or 'No' for the presence or absence of the drug(s) in urine samples. If semiquantitative values are needed, for example, as in monitoring medication levels, the field test can still be used, but it is necessary to include several standard concentrations. And a minimal quality control is necessary. More detailed studies, requiring precise quantitative information, will need more elaborate instrumentation to fulfill all criteria for HPTLC, but it may well be useful and economical to carry out a prescreening of the study group using the field method.

Applications of Laboratory-Based Methods Using (HP)TLC

Thin layer chromatographic techniques to determine CQ and DCQ have been described by several groups, (Essien & Afamefuna 1982; Essien & Ifudu, 1984; Betschart & Steiger, 1986). Using TLC, the Nigerian group studied the distribution of chloroquine and its metabolites between maternal blood, cord blood and neonatal blood. They also studied chloroquine excretion in urine. The chloroquine was determined either by using Dragendorff's reagent, or by eluting the drug from the silica gel followed by spectrophotometry.

Betschart & Steiger, (1986), described a method in which drugs are extracted from alkaline-buffered body fluids by liquid-liquid extraction using heptane/isoamylalcohol, with an extraction efficiency of >80%. Chromatographic separation was carried out on HPTLC silica gel plates with toluene/diethylamine (9/1) as the developing solution. CQ and its metabolites were detected by using a mercury lamp, and measuring fluorescence emission. The method allows the detection of 10 nmol CQ/L. It has been adapted to measure other antimalarial drugs such as quinine, mefloquine and amodiaquine in body fluids. The lowest concentrations detected are indicated in table 3. A list of the minimal essential equipment, required to exploit the full power of HPTLC, is given in Table 4.

Studies in which this method has already been used include that of Stahel et al., (1986), in which drug levels in individual patients with malaria were monitored in order to investigate the relationship between the prophylaxis scheme, the severity of resistance and the drug levels. The method has also been used to: determine the drug levels in patients with chloroquine-resistant malaria infections, (unpublished data); study drug levels in blood samples of tourists returning from

tropical countries; assess the amount of chloroquine in tablets from different origins, and compare different prophylaxis schemes, (several small doses versus one high dose weekly), and their efficiency in guaranteeing protective levels in the blood, (Lagrove, 1986).

In a larger scale study, the chloroquine consumption in a village population in Ifakara, Tanzania was probed, during annual surveys on the appearance of resistance, by analysing plasma and urine samples at the beginning of the survey. The results showed that around 50% of the children had chloroquine, (up to 30 $\mu\text{mol/L}$ urine), and its metabolites in the urine when they entered the survey. This reflects a history of considerable drug consumption in the study population, (Hatz, Betschart & Tanner, unpublished data), which has to be considered in epidemiological studies on malaria transmission and the appearance of resistance. The efficiency of chloroquine treatment was then assessed by measuring the drug concentrations in the same group of children 3 days after treatment. All had protective levels of chloroquine in the plasma, as well as high levels in the urine samples.

Outlook

A variety of thin-layer chromatographic methods have been discussed ranging from a simple field test, which can provide a fast, reliable answer for the presence of chloroquine and its metabolites, to a semiquantitative test, which allows estimation of the concentration of the drugs in the urine, to the quantitative HPTLC-technique to assay the antimalarials in blood, plasma, erythrocytes or urine. It is also possible to discriminate between chloroquine, quinine, amodiaquine and their metabolites. All the methods are very useful tools for different purposes in investigations concerning antimalarial drug consumption. Each level of the test system requires a different level of material investment, and also makes different demands on the training and competence of the staff who will use the test. In order to make the possibility of carrying out field tests widely available, the production of a small, simple test kit, which can be used anywhere, is being planned.

It is already clear that the method is valuable and has many possible applications. However, it must be borne in mind that there is still a need for many studies, for example to assess the predictive power of the test, and in particular to define for what purposes the relatively simple estimation of drug concentration in urine is adequate, and how far diurnal variation in drug concentration in the urine could affect conclusions.

Finally, it is worth emphasizing that the thin layer chromatographic methods discussed are not only useful for determinations of antimalarial drugs, but that the laboratories which invest time and money in acquiring the necessary skills and equipment will have a versatile tool at their disposal.

Acknowledgements

The laboratory work to establish the HPTLC-technique and to develop a simplified field test was carried out in the biochemistry laboratory of the Swiss Tropical Institute in Basel, (Dr. B. Betschart). The field work was carried out as a part of the Kilombero Health Research Programme, (KIHRE), in Ifakara, Tanzania (Project coordinators: Drs. M. Tanner and Ch. Hatz), and in collaboration with the National Institute of Medical Research of Tanzania (NIMR, Director General: Prof. W. Kilama), at the Amani Research Centre, (Director Dr. S.G.M. Irare). Research clearance was obtained from the Tanzania National Scientific Research Council. The technical assistance of A. Sublet and S. Steiger in the development of the field test, as well as the help of J. Jenkins in critically reading this manuscript, is gratefully acknowledged.

TABLE 1

Major Differences Between Thin Layer Chromatography (TLC)
and Column Chromatography (HPLC)

	TLC	HP(LC)
Sample	multiple, and run concurrently	single, sequential
System	open (plates)	closed (columns)
Separation	development	elution
Detection	(mostly) static, with a wide range of physical and chemical detection methods	dynamic, and limited to available detector

* (modified from Geiss, 1987)

TABLE 2

Comparison of Typical TLC and HPTLC Properties

	TLC	HPTLC
Typical plate size	20 X 20 cm	10 X 10, 10 X 20 cm
Particle size of silica sorbent	12 - 15 μ m	6 - 7 μ m
Sample size	μ l range	nI range
Sample application	manual	instrumental
Separation distance	10 - 15 cm	3 - 8 cm
Separation time	> 20 min	< 20 min.
Detection	qualitative semiquantitative	quantitative densitometry
Detection limits	microgram	micro/nanogram

Rf-values, Instrumental Detection Limits and Detection Modes
of Different Antimalarial Drugs after HPTLC with
Toluene/Diethylamine/Methanol (8:1:1)

Drug	Rf-value	Detection	
		limit	mode
Chloroquine	0.53	1 ug/L	blue fluor.
Desethylchloroquine	0.35	1 ug/L	blue fluor.
Quinine	0.42	5 ug/L	blue fluor.
Amodiaquine	0.5	50 ug/L	faint fluor
Mefloquine	0.3	5 ug/L	UV absorb.

TABLE 3

TABLE 4

Minimal Instrument Equipment Required for the
Quantitative Determination of Antimalarial Drugs
with HPTLC

- Extraction equipment (glass tubes, evaporation unit)
- Quantitative application (e.g. via Nanomat, Linomat)
- Chromatographic development unit (glass tanks or linear development chamber)
- Scanner with fluorescence detection and at least an analog writer

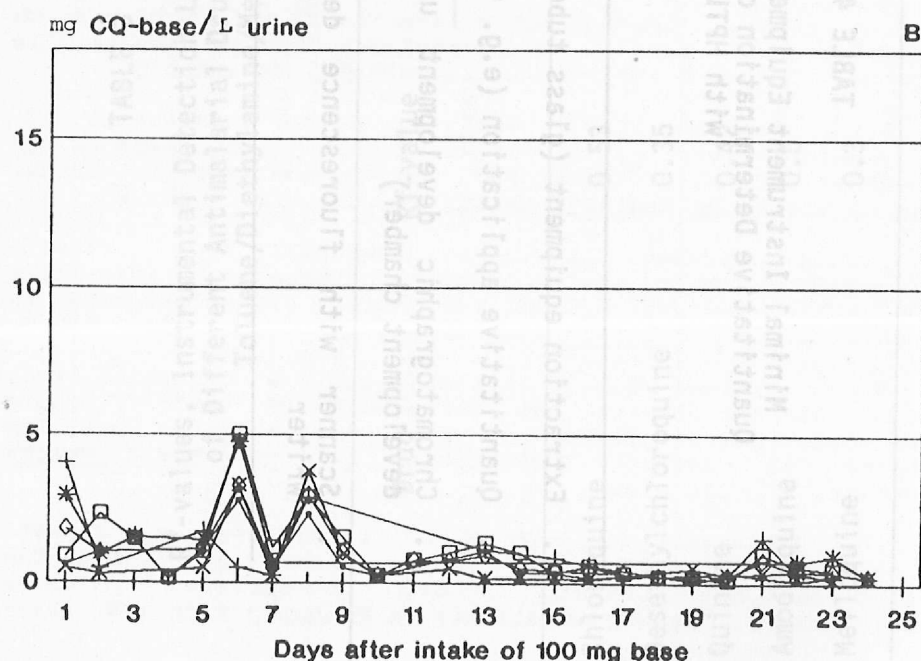
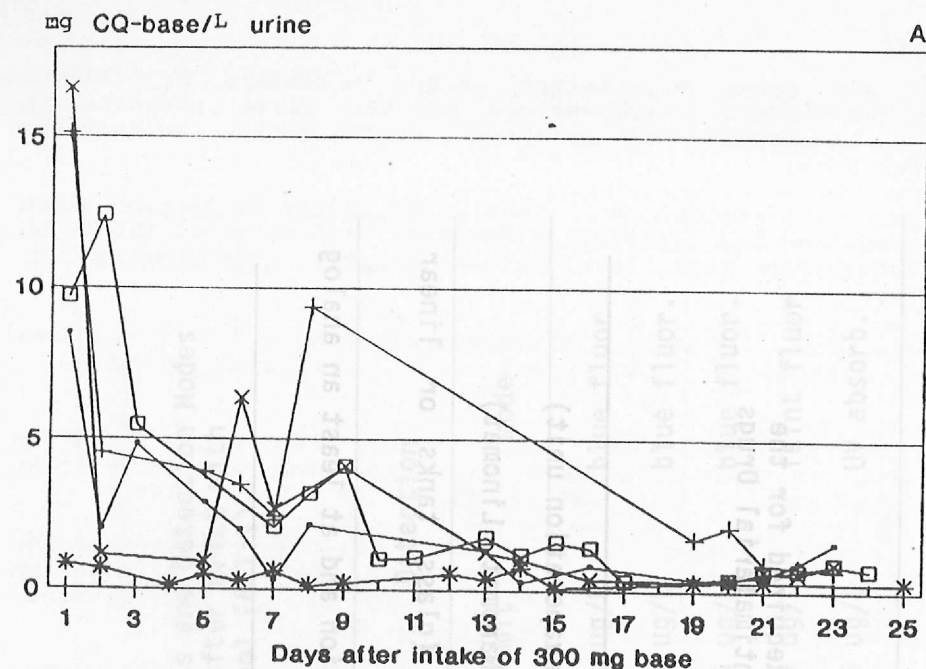


Figure 1: Chloroquine concentrations detected in daily urine samples of two groups of volunteers after a single dose of either 300 mg (A) or 100 mg (B) chloroquine base.

References

- Bertsch, W., Hara, S., Kaiser, R.E., & Zlatkis, A. (1980). Instrumental HPTLC. Proceedings of the First International Symposium on HPTLC, Bad Durkheim. Huthig Verlag, Heidelberg, Basel, New York.
- Betschart, B. & Steiger, S. (1986). Quantitative determination of chloroquine and desethylchloroquine in biological fluids by high performance thin layer chromatography. *Acta Tropica*, 43, 125 - 130.
- Churchill, F.C. (1989). Field-adapted assays for chloroquine and its metabolites in urine and blood. *Parasitology Today*, 5, 116 - 126.
- Essien, E.E. & Afamefuna, G.C. (1982). Chloroquine and its metabolites in human cord blood, neonatal blood, and urine after maternal medication. *Clinical Chemistry*, 28, 1148 - 1152.
- Essien, E.E. & Ifudu, N.D. (1984). Residual chloroquine and metabolites in man as a sequel of previous chloroquine medications: a urinary excretion study and its significance. *Journal of Tropical Medicine and Hygiene*, 87, 131 - 136.
- Geiss, F. (1987). Fundamentals of thin layer chromatography. Huthig Verlag, Heidelberg, Basel, New York.
- Lagrange, M. (1986). Concentration sanguine de chloroquine et de desethylchloroquine dans la prophylaxie antipaludéenne a la chloroquine. These, Universite Louis Pasteur, Strasbourg.
- Mount, D.L., Patchen, L.C., Williams, S.B., & Churchill F.C. (1987). Colorimetric and thin-layer chromatographic methods for field assay of chloroquine and its metabolites in urine. *Bulletin of the World Health Organization*, 65, 615 - 623.
- Mount, D.L., Patchen, L.C. & Churchill F.C. (1988). Field-adapted method for high-performance thin-layer chromatographic detection and estimation of chloroquine in finger-stick blood. *Journal of Chromatography*, 428, 196 - 202.
- Mount, D.L., Nahlen, B.L., Patchen, L.C. & Churchill, F.C. (1989). Adaptations of the Saker-Solomons test: simple reliable colorimetric field assays for chloroquine and its metabolites in urine. *Bulletin of the World Health Organization*, 67, 295 - 300.
- Stahel, E., Betschart, B., Brun R. & Lagrange, M. (1986). Selektionierung von chloroquinresistenten Plasmodium-falciparum-Stämmen bei Touristen unter Chemoprophylaxe. *Schweiz.medizinische Wochenschrift* 116, 734 - 738.

**FIELD ASSAY FOR THE SEMIQUANTITATIVE DETERMINATION
OF CHLOROQUINE AND ITS METABOLITES IN URINE
BY USING THIN LAYER CHROMATOGRAPHY**

1. Preparation of the Plates

The plates used are coated with HPTLC silica gel 60, aluminium backed, without fluorescence indicator, 10 X 20 cm. If the plates supplied are 20 X 20 cm, they can be cut in half, preferably with a paper-cutting guillotine; if not, with scissors.

Mark application spots along the two longer sides of the plate with a pencil and a ruler as schematically indicated in figure 1. This allows the application of 24 samples per plate. Number the first, and the last, application spots for identification purposes.

2. Preparation of standards

Dissolve 10 mg chloroquine diphosphate in 6 ml of a pretested blank urine sample to give a stock solution of 1.67 g/L of the diphosphate, which is equivalent to 1 g/L of chloroquine base. This stock solution has to be diluted, (see Table 1), to give appropriate concentrations on the plate for semi-quantitative evaluation. Three samples should be applied, corresponding to 1, 5 and 50 mg chloroquine base/L urine.

3. Sample Application

Apply samples of 25 µl volume using a micropipette with an Eppendorf tip. The three standards should be applied first, starting with the highest concentration. Apply the samples directly onto the predrawn marks gently, in small droplets. The pipette tip should not touch the surface of the plate. The Eppendorf tip can be reused after rinsing twice with 70% ethanol. When all the samples have been applied, allow the plate to dry in air.

4. Chromatography**A. Using a Linear Development Chamber**

The linear development chamber is placed on a horizontal surface. Use the spirit-level supplied with the chamber to check position. Fill each groove with 5 ml of the premixed developing solution, (toluene-diethylamine-methanol, 8: 1: 1 by volume). Insert the glass strips. Put the plate into the chamber with the white coated side facing

up. Press the lateral buttons to push the glass strips towards the sides of the plate. The contact between the developing solution and the plate starts the development by capillary forces. As soon as the migration starts, close the chamber. When the two solvent fronts meet in the middle of the plate, (this takes about ten minutes), remove the plate from the chamber and allow it to dry, briefly, in the air. Then soak the developed plate in a solution of 40% paraffin in hexane, to preserve the fluorescence.

B. Using Other Types of Container

If a linear development chamber is not available, chromatography can be carried out in any suitably-shaped glass vessel with a lid. In this case, samples must be applied to one edge of the plate only. Sufficient solvent is placed in the vessel to come to a level just below the spots, the plate inserted and the chamber closed. The development is stopped after 15 minutes by removing the plate from the glass vessel. To preserve the fluorescence the air dried plate is soaked in 40% paraffin in hexane as above.

5. Evaluation

The plate is examined with a hand-held UV lamp with an excitation wavelength of 366 nm. To see the fluorescence clearly, it is necessary to look at the plate in a dark place; for example it can be placed in a cardboard box covered with a black cloth. Positive samples show a blue fluorescent spot. By comparing the brightness of the samples with the standards, it is possible to estimate, semiquantitatively, the amount of the drug. For example, if the spot is intermediate in brightness between those of standards 1 and 2, one can say that the content of chloroquine base in the original urine was between 5 and 50 mg/L urine, (Table 2).

6. Use of the Method for Other Antimalarial Drugs

In order to obtain semiquantitative estimates of the concentration of quinine and amodiaquine the same procedure can be used provided the appropriate standards are included.

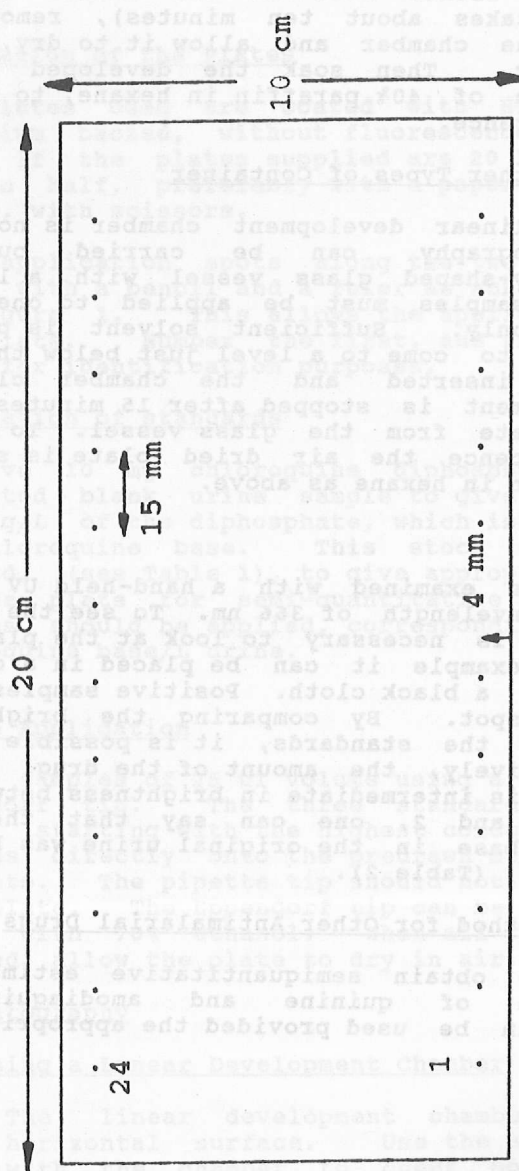


Fig.1. Scheme of a TLC plate with the distances and the marks which have to be made with a pencil before applying samples.

TABLE 1
Preparation of the Standards from a Stock Solution of Chloroquine-Diphosphate Equivalent to 1 g/L Base

Nr	Initial Stock (mg/L)	ul Stock	ul Blank Urine	Final Stock (mg/L)
1	1000	50	950	50
2	50	100	900	5
3	5	200	800	1

Table 2
Concentrations of Chloroquine Base on the Plate After Applying 25 ul of the Three Standard Solutions

Nr	Volume	(CG) on Plate	(CQ) in Urine
1	25 ul	1250 ng	50 mg/L
2	25 ul	125 ng	5 mg/L
3	25 ul	25 ng	1 mg/L

Data sheet:

A = more than 50 mg/L urine
B = between 5 and 50 mg/L urine
C = between 1 and 5 mg/L urine
D = less than 1 mg/L

PLATE No:

PLATE No:

SAMPLE	A	B	C	D	SAMPLE	A	B	C	D
1.	1.
2.	2.
3.	3.
4.	4.
5.	5.
6.	6.
7.	7.
8.	8.
9.	9.
10.	10.
11.	11.
12.	12.
13.	13.
14.	14.
15.	15.
16.	16.
17.	17.
18.	18.
19.	19.
20.	20.

LIST OF MATERIAL REQUIRED FOR THE
HP(TLC) FIELD TEST

The items indicated in the following list have already successfully tested under laboratory and field conditions. companies mentioned in the list are not necessarily the excl suppliers of all these items. Other products might be used as but it is recommended that they are first tested out.

- HPTLC aluminium foils coated with silica gel 60 (20 x 2 without fluorescence indicator, package with 25 foils. Nr. 5547, MERCK, Darmstadt, BRD.
- Toluene Nr. 8331 MERCK, Darmstadt, W. German
Methanol Nr. 6008 MERCK, Darmstadt, W. German
Diethylamine Nr. 803010 MERCK, Darmstadt, W. German
Paraffin, liquid Nr. 7162 MERCK, Darmstadt, W. German
Hexane Nr. 4368 MERCK, Darmstadt, W. German
- Chloroquine diphosphate Nr. C-6628 SIGMA, St. Louis, Mo.
Quinine, free base Nr. Q-1625 SIGMA, St. Louis, Mo.
Desethylchloroquine was kindly provided by STERLING- WINT Rensselaer, NY 12144, USA.
- HPTLC linear development chamber 10 x 20 cm, complet 022.8505, CAMAG, Sonnenmattstrasse 11, Muttenz, Switzerland
- Hand lamp, with solar panel and rechargeable batte commercially supplied with a daylight bulb. CHRONAR C Princeton, NJ 08542, USA.
- Long wave mercury tube 366 nm, Nr. 352.00132, C Sonnenmattstrasse 11, Muttenz, Switzerland.
- Micropipette, 10 - 100 ul and yellow tips e.g. from Eppe GmbH, Hamburg, BRD.

A REVIEW OF THE DETECTION LEVELS ATTAINABLE WITH
CURRENT ANALYTICAL METHODS FOR ANTIMALARIALS
IN BODY FLUIDS

Ailene Jamaludin & Visweswaran Navaratnam

INTRODUCTION

The emergence of resistant strains of *Plasmodium falciparum* to many of the synthetic antimalarials like chloroquine, has led to a reappraisal of many of the older antimalarials and also a search for new ones. A more systematic approach to the choice of doses, and dosing regimens, are pursued to optimise the therapy of malaria. One way to reach this objective is to get as much information as possible on the pharmacokinetic behavior of these compounds in man. In the past, the unavailability of sensitive and specific analytical methods has led to the paucity of pharmacokinetic information based on the detection of low concentrations in plasma or urine. This paper is a review of the more sensitive analytical methods available today and aims at providing an awareness of the currently available detection limits. The methods that are considered superior are those that are practical, and feasible, for routine analysis in body fluids.

CHLOROQUINE AND OTHER 4-AMINOQUINOLINES

The analyses of chloroquine in body fluids were accomplished, in the past, by several spectrophotofluorometric methods, (Brodie et al., 1947; McChesney et al., 1956 and Adelusi and Salako, 1980). However, these methods were not sufficiently sensitive and, also, could not resolve chloroquine from its metabolites, e.g. desethyl chloroquine. This was also true for the other 4-aminoquinolines such as amodiaquine and hydroxychloroquine.

The most specific and sensitive methods, reported to date for chloroquine, are HPLC methods, (Pussard et al., 1986; Williams et al., 1988; Mihaly et al., 1985; Alvan et al., 1982; Bergqvist and Holmberg, 1980) and one GC method reported by Kuye et al., 1983. The latter process was rather complex with the need to form chloroformates of chloroquine, and prior examination of the chloroformate products by TLC, before analysis by GC.

The more practical and appropriate methods for analysis of chloroquine from body fluids are the HPLC methods, with either UV or fluorescence detection. Extractions of chloroquine, hydroxychloroquine, amodiaquine and desethylchloroquine, (a metabolite of chloroquine), from plasma, whole blood or urine have been conducted with organic solvents like methylene dichloride, hexane-methyl-tert-butyl ether (1:1) mixtures,

diethyl ether, ethylene dichloride and n-heptane. Table 1 shows the more sensitive HPLC methods reported for the analysis of 4-aminoquinolines from body fluids.

Detection Levels for the Compounds of Interest, With the Available Analytical Methods, Were Fairly Sensitive, With Concentrations Ranging from 1 to 8 ug/L for Chloroquine.

The most sensitive analytical method to date is the method reported by Alvan et al., (1982). They used a chromatographic system consisting of a Waters M45 pump, a U6K manual injector and a Spectra-Physics 970 fluorescence detector. The excitation and emission wavelengths are 335 nm and 370 nm, respectively. The analytical column used was slurry packed with LiChrosorb Si 60 of particle size 5 μ m. The dimensions of the column was 0.15 m X 4.6 mm i.d. The mobile phase comprised of acetonitrile-methanol-diethylamine, (80:19.5:15), at a flow rate of 1 ml/min.

The extraction procedure developed by Alvan et al., (1982), could be used for plasma, red blood cell assay and urine samples. For plasma samples, 1 ml of 1M sodium hydroxide was added before extraction with diethylether. The ether phase was then further dried with 0.2 - 0.3 g sodium sulfate. This was done to remove any water in the system which could have been detrimental to the analytical column. The ether was removed after centrifugation and then evaporated at 30°C under a gentle stream of nitrogen. The dried residue was reconstituted with 200 μ l of the mobile phase, passed through a pasteur pipette, closed at one end with glass wool, and a 100 μ l aliquot was injected into the HPLC.

The percentage recovery of chloroquine from plasma, red blood cells and urine, was 88%, 80% and 88%, respectively. Detection limits obtained with this method were 1 μ g/L of plasma for chloroquine and 0.5 μ g/L for desethyl-chloroquine. However, day to day, and within day, variation were not reported.

QUININE AND QUINIDINE

In the past, the analysis of quinine or quinidine in body fluids were conducted with fluorescence methods which lack specificity and sensitivity, (Spinks and Tottey, 1984 and Cramer and Isaksson, 1963). More recent methods, which use HPLC, were able to improve both specificity and sensitivity, (Edstein et al., 1983; Ochs et al., 1980; Barrows et al., 1980; and Arunyanart and Cline Love, 1985). A gas chromatography-mass spectrometry, (GC-MS), has also been reported by Furner et al., (1981), but this required expensive instrumentation and was too complex for routine work.

Table 1: Summary of Analytical Methods and Detection Levels for 4-aminoguanolines in Body Fluids

Compound	Author	Method of Analysis	Extraction Solvent	Buffer pH	Column	Mobile phase	Limit of detection
Chloroquine (CQ) Amodiaquine (AQ)	Pussard et al (1986)	HPLC reverse phase with UV detector at 340 nm	Methylene dichloride	Dipotassium hydrogen phosphate (pH 9.5)	Water C ₁₈ Novapak, 5 µm particle size; 15 X 0.39 cm	45mM potassium dihydrogen phosphate buffer adjusted to pH3 with orthophosphoric acid and 12% acetonitrile flow-rate - 0.6 ml/min	3.2 µg/L - CQ 3.6 µg/L - AQ (1 ml plasma, and whole blood and 500 ul urine).
Hydroxy-chloroquine (HCQ) Chloroquine (CQ)	Williams et al (1988)	HPLC normal phase with fluorescence detector. Excitation 320 nm Emission 380 nm	hexane: MTBE (1:1) MTBE methyl tert-butyl ether		DuPont Zorbax-Sil column, 5 µm particle size	Hexane-MTBE-methanol (1:1:1) containing 0.5% n-butylamine. Flow rate - 1 ml/min	10 µg/L - HCQ 8 µg/L - CQ (100 ul whole blood or urine)
Amodiaquine (AQ)	Mihaly et al. (1985)	HPLC reverse phase with UV detector at 340 nm	Diethyl ether		uBondapak Rad-Pak phenyl, 10 µm particle size 10 cm X 8 mm	Water:methanol (73:27, v/v) containing triethylamine (1%) adjusted to pH 2.8 with orthophosphoric acid	5 µg/L (1 ml of plasma)

Cont. Table 1

Compound	Author	Method of Analysis	Extraction Solvent	Buffer pH	Column	Mobile phase	Limit of detection
Chloroquine (CQ) and desethyl - chloroquine (DCQ)	Alvan et al (1982)	HPLC with fluorescence detection. Excitation - 335 nm Emission - 370 nm	diethyl ether	1M sodium hydroxide	Merck Lichrosorb Si60 with packing material of particle size 5 µm and dimensions 0.15 m X 4.6 mm i.d.	acetonitrile - methanol - diethylamine (80 : 19.5 : 0.5) at a flow rate 1 ml/min	1 µg/L - CQ 0.5 µg/L - DCQ
Chloroquine and desethyl - chloroquine	Bergqvist and Holmberg (1980)	HPLC with fluorescence detection at excitation wavelength of 335 nm and emission 370 nm or with ultraviolet detection at 254 nm or 340 nm	ethylene dichloride	1M sodium hydroxide	Nucleosil C ₁₈ , 5 µm particle size and dimensions 200 X 4.0 mm i.d.	acetonitrile - phosphate buffer (40:60) (pH 3 and perchlorate 75 mmol/L) at a flow rate of 0.8 to 1 ml/min	0.5 n mol/L for CQ and DCQ from 500 ul of plasma or 200 ul of urine

Cont. Table 1

Compound	Author	Method of Analysis	Extraction Solvent	Buffer pH	Column	Mobile phase	Limit of detection
Chloroquine and desethyl chloroquine	Brown et al (1982)	ion-pair HPLC reversed-phase with UV detection at 340 nm.	n-Heptane	IN sodium hydroxide	Waters C ₁₈ uBondapak, packing material and particle size 10 µm and dimensions 300 mm X 3.9 mm i.d.	0.02M 1-heptane sulfonic acid and acetonitrile. A pumping ratio of 66:34 of PIC 8-7 reagent to acetonitrile in an isocratic mode.	5 ng for CQ and DCQ from 1 ml of plasma.

The HPLC methods developed for quinine and quinidine with UV, or fluorescence, detection produced limits of detection that are adequate to perform pharmacokinetic studies, (see Table 2).

The Detection Levels for Quinine and Quinidine, With the Available Analytical Ranged from 10 - 50 µg/L from Plasma

The reported extraction solvents used for extracting quinine and quinidine from body fluids were, hexane:ethyl acetate (9:1, Mihaly et al., 1987) and dichloromethane:isopropanol (4:1, v/v by Guentert et al., 1980). Leroyer et al., (1982), and Reece and Peikert (1980), did not extract the plasma samples but just precipitated the plasma proteins with acetonitrile.

The HPLC-UV method by Mihaly et al., (1987), consisted of a Waters Model 6000A solvent delivery system, a Waters U6-K manual injector and a Waters Model 481-LambdaMax variable wavelength ultraviolet detector, set at wavelength 254 nm. The analytical column used was a Waters Rad-Pak uBondapak C₁₈, with 10 µm packing and with dimensions 100 mm x 8 mm i.d. The mobile phase consisted of water:acetonitrile, (91:9, v/v), containing 1% triethylamine and adjusted to pH 2.5 with orthophosphoric acid. The flow rate of the mobile phase was 3.5 ml/min. The compounds of interest were extracted from plasma as follows: to 1 ml of plasma, was added 150 µl of an aqueous solution of monoethylglycinexylidide, the internal standard, (15 µg). The mixture was then extracted with 10 ml of hexane-ethyl acetate, (9:1). The organic layer was evaporated at 35°C under a gentle stream of nitrogen. The dried residue was reconstituted with 100 µl of the mobile phase and 40 µl was injected into the HPLC.

The recoveries of quinine and quinidine were relatively low with mean recoveries of 63% and 65%, respectively. The limit of detection however was fairly good at 10 µg/L with 1 ml of plasma. The within-day, and day to day, variations were small at less than 5% at a concentration of 1000 µg/L. Within-day variation at 10 µg/L was less than 10%.

The method of Leroyer et al., (1982), did not involve extraction of quinidine from plasma, but used direct protein precipitation of the samples with acetonitrile. Quinine was employed as the internal standard, suggesting that the method is also suitable for the analysis of quinine. The HPLC chromatographic system used here comprised of a Chromatem apparatus with an Altex 210 pump, a Rheodyne 7010 manual injector and a Schoeffel FS 970 spectrofluorometer. The excitation and emission wavelengths were set at 340 nm and 418 nm, respectively.

Table 2: Summary of Analytical Methods and Detection Levels of Quinine and Quinidine in Body Fluids

Compound	Author	Method of Analysis	Extraction Solvent	Buffer pH	Column	Mobile Phase	Limit of Detection
Quinine (QN) and Quinidine (QND)	Mihaly et al (1987)	HPLC reverse phase with a UV detector at 254 nm	Hexane:ethyl acetate (9:1)		Rad-Pak uBondapak C ₁₈ ; 10 µm particle size; 100 X 8 mm i.d.	Water:acetonitrile (91:9, v/v) containing triethylamine (1%) adjusted to pH 2.5 with ortho-phosphoric acid Flow rate - 3.5 ml per min.	10 µg/L for both QN and QND (1 ml of plasma)
Quinidine	Leroy et al (1982)	HPLC reverse phase with fluorescence detector Excitation - 340 nm Emission - 418 nm	Protein precipitation with acetonitrile		Water C ₁₈ , uBondapak column; 30 cm X 3.9 mm; particle size 10 µm	acetonitrile: acetic acid: water (10:4:86) Flow rate - 2.5 ml/min	50 µg/L (100 µl of plasma).

Cont. Table 2

Compound	Author	Method of Analysis	Extraction Solvent	Buffer pH	Column	Mobile Phase	Limit of Detection
Quinidine	Guentert et al (1980)	HPLC reverse phase with fluorescence detector for plasma Excitation - 254 nm Emission - 340 nm and with ultra violet detector for urine samples	dichloro-methane: isopropanol (4:1, v/v)	0.6M borate buffer pH 9.0	alkyl phenyl uBondapak particle size 10 µm; 30 cm X 3.9 mm i.d.	0.05 M phosphate buffer pH 4.5: acetonitrile: tetrahydrofuran (80:15:5, v/v) Flow rate - 1.5 ml/min	10 µg/L for quinidine (1 ml plasma) For urine samples - detection limits are not stated.
Quinidine	Reece and Peikert (1980)	a. HPLC reverse phase with fluorescence detector Excitation - 320 nm Emission - 418 nm b. GLC with an alkali flame detector	Protein precipitation with acetonitrile Chloroform	IN aqueous sodium hydroxide	Waters uBondapak alkyl phenyl reversed-phase column, 10 µm packing material particle size Supelco 3% OV-17 on Gas-chrom Q of dimensions 1 m X 2 mm i.d.	1.5M aqueous phosphoric acid and acetonitrile (90:10) at a flow rate of 2 ml/min	16 µg/L (20 µl of plasma) not stated

The analytical column was a Waters C₁₈ uBondapak column, of dimensions 30 cm x 3.9 mm i.d., and with packing material of particle size 10 μ m. The mobile phase was a mixture of acetonitrile-acetic acid-water, (10:4:86), and was used at a flow rate of 2.5 ml/min. In the preparation of the samples, 100 μ l of acetonitrile was used to precipitate the proteins in 100 μ l of plasma. The solution was then vortexed for 1 minute and then centrifuged for 10 minutes at 1,200 g. Ten μ l of the clear supernatant was then injected into the HPLC.

The recovery of quinidine was 100 \pm 3%, showing that the protein precipitation did not influence the recovery of quinidine. With 100 μ l of plasma, the minimum detection level of quinidine was 50 μ g/L. The within-day variation at concentrations, ranging from 0.5 to 5 mg/L, was good at a value of less than 3%. The day to day variation ranged from 3.3 to 6.7% at concentrations ranging from 0.5 to 5.0 mg/L.

The available analytical methods for quinine and quinidine are adequate for measuring concentrations of these compounds in body fluids.

MEFLOQUINE

Mefloquine is a 4-quinoline methanol derivative and its hydrochloride salt is a white, odorless and bitter tasting powder. The various analytical methods and their detection levels for mefloquine are listed in Table 3. The concentrations of mefloquine in body fluids are in the low microgram range, as demonstrated in single dose studies by Desjardins et. al, (1979), and Schwartz and Renalder, (1981). Therefore, this sensitivity, requirement excludes the use of the HPLC method by Grindel et al. (1977), and the TLC method of Schwartz et al., (1980), with detection limits of 50 and 100 μ g/L, respectively.

The Limits of Detection for Mefloquine Ranged from 1 μ g/L to 100 μ g/L from 1 ml of Plasma, and 5 μ g/L from 0.5 ml of Plasma

The HPLC method reported by Kapetonovic et. al, (1983), had a low detection limit of 10 μ g/L, using a starting plasma volume of 1 ml. However, the extraction procedure was quite lengthy and poor extraction recoveries were reported.

The most popular method for the determination of mefloquine is gas liquid chromatography with electron-capture detector, (GLC-ECD). Nakagawa et. al, (1979), was able to detect 10 μ g/L mefloquine with the ECD, but only 100 μ g/L with the flame ionization detector, (FID). This is probably due to the presence of the two trifluoromethyl groups at position two, and eight, of the mefloquine molecule which enhances electron capture detectability, and enables detection at very low microgram levels.

Table 3: Summary of Analytical Methods and Detection Levels for Mefloquine in Body Fluids

Author	Method of Analysis	Detector Used	Extraction Solvent	Buffer pH	Column	Mobile Phase	Limit of Detection
Grindel et al. (1977)	HPLC	UV detection 280 nm	ethyl acetate	Phosphate Buffer (pH 7.4)	10 μ m silica-cyanopropyl silane	IPE-dioxane-acetic acid (3:2 + 0.5%)	50 μ g/L (5 ml) plasma
Schwartz et al. (1980)	TLC	300 nm	isopropyl acetate	TRIS Buffer (pH 8)		Dichloro-methane:MeOH:Acetic Acid (80:10:10)	100 μ g/L (1 ml) plasma
Schwartz and Renalder (1981)	GC-MS		isopropyl acetate	Glycine-NaCl (pH 9)	3% SE 30		1 μ g/L (1 ml) plasma or whole blood

Author	Method of Analysis	Detector Used	Extraction Solvent	Buffer pH	Column	Mobile Phase	Limit of Detection
Nakagawa et al. (1978)	GLC	ECD or FID	ethyl acetate and Diethyl ether	0.1N NaOH	3% phenyl methyl silicone	-	10 ug/L and 100 ng/ml (5 ml sample)
Kapetonovic et al. (1983)	HPLC	UV detection 222 nm	ethyl acetate-hexane (3:2) or MeOH or dichloromethane	0.5 M dibasic sodium phosphate or 0.5 M monobasic Amm. Phosphate or 0.2 M perchloric acid	uBondapak C ₁₈	Methanol-water	10 ug/L (1 ml plasma)
Hiezmann & Geschke (1984)	GLC	ECD	Dichloromethane	TRIS Buffer (pH 8)	3% SP-2250	-	5 ug/L (0.25 ml plasma)
Dadgar et al. (1985)	GLC	ECD	isopropyl acetate	glycine-NaCl (pH 9)	3% GE-SE-30	-	10 ug/L (1 ml plasma)

The analysis of mefloquine by TLC and HPLC, requires large volumes of plasma, and lengthy extraction procedures. Also it is not sensitive enough to quantify the lower levels of mefloquine in body fluids.

Heizmann and Geschke (1984), has reported a procedure which achieved a detection limit of 5 ug/L with only 0.25 ml of plasma. Derivatization was performed with trimethylsilyl-imidazole and dichloromethane was the extraction solvent.

Schwartz and Renalder, (1981), has reported the most sensitive procedure, with a detection level of 1 ug/L, by using Gas Chromatography with Mass Spectrometry (GC-MS). This assay can detect 1 ug/L mefloquine in plasma and 3 ug/L in whole blood and urine. However, this method requires expensive instrumentation, which is not available in most laboratories, and the cost is too high for routine work.

The method of Heizmann and Geschke (1984), was repeated in our laboratory, (Centre for Drug Research, Universiti Sains Malaysia, Penang) with some modifications.

The extraction procedure was carried out as follows:

- To 50 ng of the internal standard. [DL-(2-pyridyl)-2,7-bis (trifluoromethyl)-4-quinoline methanol], was added 200 ul of the plasma sample.

- Tris buffer, (200 ul at pH 8.0), was then added before extracting twice, (7 ml x 2), with dichloromethane.

- The lower dichloromethane layer was aspirated and evaporated to dryness, at 60°C, under a gentle stream of nitrogen. A 100 ul of a 10% solution of trimethylsilylimidazole (TSM) was added to the dried residue.

- After stirring, the solution was left to stand at room temperature (28°C) for 15 minutes.

- The resultant mixture was diluted with 500 ul acetonitrile, and 1 ul was injected into the GLC.

- The chromatographic system comprised of a HP 5880A Gas Liquid Chromatography instrument, with a electron capture detector consisting of 15 mCi nickel-63 (GLC-ECD).

- The GLC-ECD was operated under the conditions listed below:

Detector	: Electron-capture detector (ECD) Ni ⁶³
Column	: 3% OV-17 on 100/120 chromosorb HP
Detector temperature	: 280°C
Injector	: 320°C
Column oven temperature	: 185°C
Program rate	: 8°C per min
Carrier gas (nitrogen)	: 30 ml/min
Initial time	: 4 min
Final time	: 4 min
Equilibrium time	: 3 min
Run time	: 14.87 min

Figure 3.1 shows the chromatogram of plasma extracts of mefloquine. The chromatogram showed that some contaminants appear at the initial, and later stages, of the chromatogram, but they were well resolved from the peaks of interest. This showed that the dichloromethane extracts were free from interfering contaminants, and the GLC-ECD conditions gave good separation.

The mean percent recovery for mefloquine from plasma was 95% at concentrations ranging from 39 to 10,000 ug/L. The mean within day variation of mefloquine from plasma was 3.56% at concentrations ranging from 25 to 2,000 ug/L, while the mean day to day variation was 3.40% at concentrations ranging from 100 to 1,500 ug/L.

The assay procedure could reliably detect concentrations of 5 ug/L, and above, with 200 ul plasma sample, while lower concentrations were not detected. If necessary, however, the limit of detection can be further reduced by using a larger volume of plasma sample, or by decreasing the final dilution volume of the dried residue.

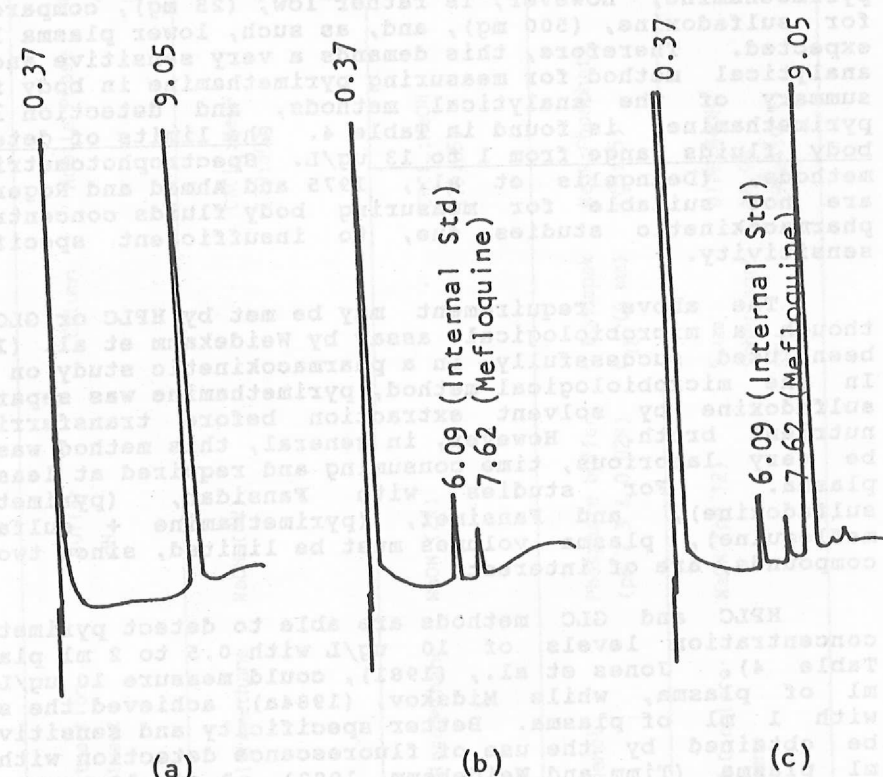


Figure 3.1: The chromatogram of Mefloquine analysis.
 (a) Dichloromethane extract of blank plasma.
 (b) Methanolic standard containing 50 ng internal standard and 50 ng Mefloquine.
 (c) Dichloromethane extract of plasma containing 50 ng internal standard and 50 ng Mefloquine.

PYRIMETHAMINE

Pyrimethamine is a 2,4-diaminopyrimidine derivative. Pyrimethamine is normally used in conjunction with sulfadoxine, (Fansidar), for malaria chemotherapy. The therapeutic dose of pyrimethamine, however, is rather low, (25 mg), compared to that for sulfadoxine, (500 mg), and, as such, lower plasma levels are expected. Therefore, this demands a very sensitive and specific analytical method for measuring pyrimethamine in body fluids. A summary of the analytical methods, and detection levels for pyrimethamine, is found in Table 4. The limits of detection in body fluids range from 1 to 13 ug/L. Spectrophotometric and TLC methods, (DeAngelis et al., 1975 and Ahmad and Rogers, 1980,) are not suitable for measuring body fluids concentrations in pharmacokinetic studies due, to insufficient specificity and sensitivity.

The above requirement may be met by HPLC or GLC methods, though a microbiological assay by Weidekamm et al. (1982), has been used successfully in a pharmacokinetic study on Fansidar. In the microbiological method, pyrimethamine was separated from sulfadoxine by solvent extraction before transferring to a nutrient broth. However, in general, this method was found to be very laborious, time consuming and required at least 2 ml of plasma. For studies with Fansidar, (pyrimethamine + sulfadoxine), and Fansimef, (pyrimethamine + sulfadoxine + mefloquine), plasma volumes must be limited, since two or three compounds, are of interest.

HPLC and GLC methods are able to detect pyrimethamine at concentration levels of 10 ug/L with 0.5 to 2 ml plasma. (see Table 4). Jones et al., (1981), could measure 10 ug/L, using 2 ml of plasma, while Midskov, (1984a), achieved the same limit with 1 ml of plasma. Better specificity and sensitivity could be obtained by the use of fluorescence detection with only 0.5 ml plasma (Timm and Weidekamm, 1982). A simultaneous assay for pyrimethamine, sulfadoxine and N₄-acetyl sulfadoxine (metabolite of sulfadoxine) was reported by Midskov (1984b) and Edstein (1984). However, the method by Edstein however showed lower percent recovery of pyrimethamine, (85.6%), when compared to other published methods, while the extraction procedure of Midskov was rather lengthy, even though good sensitivity was obtained.

The reported GLC-ECD procedures also show good sensitivity and specificity. The presence of the chlorophenyl group at position 5 of the pyrimethamine molecule enhanced the detectability of pyrimethamine by GLC-ECD, and good chromatographic analysis was obtained without the need for derivatization. Hence, is just as simple as with the HPLC methods.

Table 4: Summary of Analytical Methods and Detection Levels for Pyrimethamine in Body Fluids

Author	Method of Analysis	Detector Used	Extraction Solvent	Buffer pH	Column	Mobile Phase	Limit of Detection
DeAngelis et al. (1975)	TLC	Fluorescence 295 nm	Dichloromethane	NaOH (8N)	-	CHCl ₃ :MeOH (70:30)	5-10 ug/L ml (1.5 ml plasma)
Simmons and DeAngelis (1983)	TLC	Fluorescence 300 nm	Dichloromethane	NaOH (2N)	-	CHCl ₃ :MeOH (75:25)	10 ug/L (2 ml plasma)
Levin et al. (1978)	HPLC	UV detection 280 nm	Methanol	Phosphate buffer (pH 7.3 & 0.02N)	u Bondapak C ₁₈ (4 mm)	MeOH:Phosphate (65:35)	-
Jones and Ovenell (1979)	HPLC	UV detection 254 nm	1, 2-Dichloro-methane	NaOH (pH 12)	5 um Spherisorb SSW	DIPE:MeOH: NH ₄ OH (96:4:0.1)	10 ug/L (2 ml plasma)

Cont. Table 4

Jones et al. (1981)	GLC	ECD	toluene	NaOH (0.1M)	OV-17		5 ug/L (1 ml plasma)
Timm and Weidekamm (1982)	HPLC	Fluorescence 290 nm	n-butylchloride- dichloromethane (96:4)	NaOH (2N)	5 um Lichrosorb SI 60	MeOH:CH ₃ CN: NH ₃ -DIPE (6:25:0.1:71)	10 ug/L (0.5 ml plasma)
Author	Method of Analysis	Detector Used	Extraction Solvent	Buffer pH	Column	Mobile Phase	Limit of Detection
Edstein (1984)	HPLC	UV detection 254 nm	ethylene chloride	Phosphate Buffer (pH 3.4)	10 um Bondapak C ₁₈	MeOH:CH ₃ CN: H ₂ (25:15:60)	5 ug/L (0.5 ml plasma)
Midskov (1984a)	GLC	ECD	Dichloromethane	NaOH (0.15M)	OV-17		5 ug/L (1 ml plasma)

Cont. Table 4

Midskov (1984b)	HPLC	UV detection 280 nm	Diethyl ether and Dichloromethane	Borate Buffer (pH 10)	5 um Spherisorb S5	1. CH ₃ CN: phosphate buffer (50:50) (pH4, 0.1M) 2. CH ₃ CN: phosphate buffer (pH5.4, 0.1M) (30:70 & 20:80)	1 ug/L (1 ml plasma)
Bonini et al. (1981)	GLC	NPD					10 ug/L
Weidekamm et al. (1982)	Microbio- logical assay		Chloroform	NaOH (pH 11)			13 ug/L (2 ml plasma)

Detection limits of 50 nanogram, (on column), was achieved with the use of the GLC-ECD method by Jones et al., (1981), while Midskov, (1984a), reported a detection limit of 5 ug/L when 1 ml plasma samples were used. Bonini, (1981), used a GLC-NPD, (nitrogen phosphorus detector), for the determination of pyrimethamine and could detect a minimum of 10 ug/L. However, the method required a lengthy process when compared with that of Jones et al., (1981), or Midskov, (1984a).

The HPLC and GLC-ECD method of analysis of pyrimethamine was therefore reviewed, and assessed, by our laboratory to further improve analysis of pyrimethamine. In the assessment of the HPLC methods reported, it was possible to achieve the sensitivity limits reported. But at low concentrations, poor reproducibility was obtained when UV detection was used. In contrast to this finding, it was found that, with GLC-ECD methods, relatively good baselines and reproducibilities were obtained.

However, many practical problems surfaced with the reported extraction steps and a new procedure based on GLC-ECD was developed.

GLC-ECD Analysis of Pyrimethamine

The extraction procedure developed at our Centre was carried out as follows:

- To 200 ul of chloroquine diphosphate solution in methanol, (2 ug; the methanol was evaporated off before addition of plasma), was added 500 ul plasma extract and 500 ul potassium chloride, with a pH of 9.5.
- The mixture was then extracted twice with 6 ml aliquots of diethylether.
- The organic layers obtained were pooled and evaporated to dryness, at 60°C, under a gentle stream of nitrogen.
- The dried residue was then reconstituted with 100 ul methanol and 1 ul injected into the GLC-ECD, which was operated under the following conditions:

Detector	: Electron-captive detector Ni ⁶³
Column	: 3% OV-17 on 100/120 chromosorb
Detector temperature	: 350°C

Injector temperature	: 300°C
Column oven temperature	: 220°C
Oven temperature program rate	: 10°C
Carrier gas (nitrogen)	: 30 ml/min
Initial time	: 5 min
Final time	: 4 min
Equilibrium time	: 3 min
Run time	: 14 min

Organic solvents, such as dichloromethane, chloroform, toluene and diethyl ether, have been used for solvent extraction. However, diethyl ether was chosen here as it enabled better extraction reproducibility, and its low boiling point enabled evaporation to dryness to be achieved rapidly, thereby reducing overall analysis time.

The acid dissociation constant, (pKa), for pyrimethamine is 7.0, (Martindale, 1982), and buffering of the plasma above pH 9.00 will ensure that pyrimethamine remains in its un-ionized state, and facilitate extraction by organic solvents. In our procedure, the addition of potassium chloride saturated buffer, (pH 9.5), enabled reproducible recoveries of pyrimethamine from plasma of 92 - 95%, with a mean of 93.75%. The KCl salt resulted in improved recovery due to the salting-out effect, (Smith and Stewart, 1981). Therefore, the recoveries obtained here were higher than those reported by Jones et al., (1981), but were similar to those of Midskov, (1984a).

The mean within-day variation of pyrimethamine from plasma samples was 3.95% at concentrations ranging from 20 to 800 ug/L, while the mean day to day variation was 3.77% at concentrations ranging from 40 to 1600 ug/L.

The minimum detection limit obtained with 0.5 ml plasma was 10 ug/L. Figure 4.1 shows the chromatograms of pyrimethamine.

SULFADOXINE

The major sulfonamides used to treat malaria are sulfadoxine, sulfalene and sulfamethoxazole. These compounds are normally combined with 2,4-diaminopyrimidine for malaria treatment or prophylaxis. Sulfonamides are soluble in ethanol and chloroform, and sparingly soluble in diethylether. They are

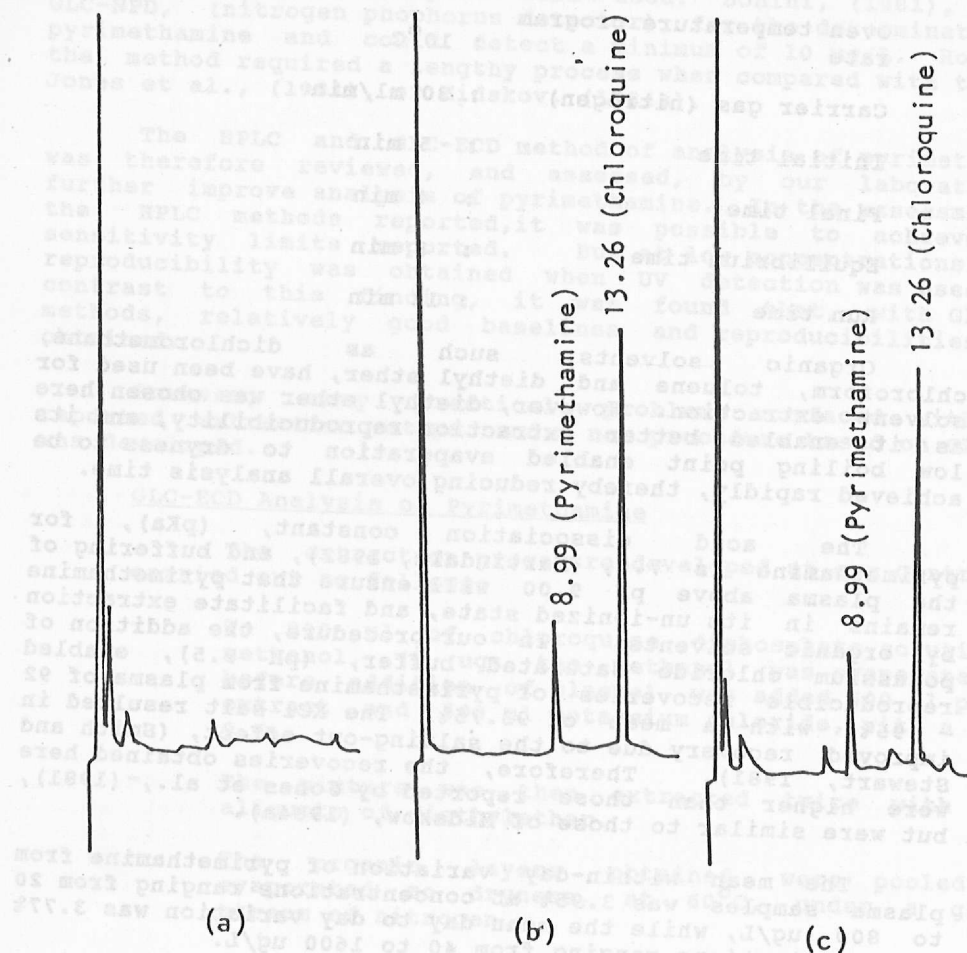


Figure 4.1: The chromatogram of Pyrimethamine analysis.
 (a) Diethylether Extract of Blank Plasma
 (b) Methanolic Standards containing 40 ng Pyrimethamine and 2 ug Chloroquine
 (c) Diethylether Extract of Plasma containing 40 ng Pyrimethamine and 2 ug Chloroquine

usually extracted from aqueous acetic solutions by organic solvents, (Clarke, 1978). The acid dissociation constant, (pK_a), of sulfadoxine is approximately 2.0, (Helboe and Thomsen, 1977), and most extraction procedures reported are extractions from aqueous acidic solutions, (See Table 5). However, Midskov, (1984), used tetrabutylammonium reagent to extract sulfadoxine by ion-pair extraction. Table 5 also shows the various published analytical methods for sulfadoxine. The minimum concentrations of sulfadoxine, in body fluids, ranged from 10 ug/L to 1 mg/L.

The traditional spectrophotometric method of Bratton and Marshall, (1939), was not specific, or sensitive, enough for sulfadoxine analysis in body fluids.

The analysis of sulfadoxine by GC has been reported by Gyllenhaal et al., (1978), and Fagerlund and Hartvig, (1979). Derivatization of sulfadoxine was necessary, with extractive alkylation being the most common form of derivatization. Bonini et al., (1981), developed a GLC-NPD procedure which simplified the derivatizing procedure, though the extraction procedure was rather lengthy and tedious.

In contrast, HPLC analysis of sulfadoxine did not require any derivatization and has been demonstrated to be sufficiently selective, and sensitive enough, for use in pharmacokinetic studies by Edstein, (1984), and Midskov, (1984).

However, the method by Edstein, (1984), did not resolve sulfadoxine from its metabolite, N_4 -acetyl sulfadoxine, and the method by Midskov, (1984), though sufficiently sensitive and specific, required a rather lengthy extraction procedure and large volumes of plasma samples.

Therefore, our laboratory took the task of developing a specific, and sensitive, assay for sulfadoxine which could be used in a pharmacokinetic study to be conducted by our centre. An assessment of available methods led us to develop an HPLC method for sulfadoxine in plasma.

HPLC Analysis of Sulfadoxine

The extraction procedure developed here was as follows:

- To 500 ul plasma was added 500 ul phosphate buffer (pH 3.4).
- The mixture was then extracted with 6 ml of a mixture of diethylether and ethyl acetate (1:1, v/v).

Table 5: Summary of Analytical Methods and Detection Levels for Sulfadoxine in Body Fluids

Author	Method of Analysis	Detector Used	Extraction Solvent	Buffer pH	Column	Mobile Phase	Limit of Detection
Helboe & Thomsen (1977)	HPLC	230 nm			LiChrosorb RP-8	CH ₃ CN:0.05M phosphoric acid	
Bonini et al. (1981)	GLC	NPD					10 ug/L
Weidekamm et al. (1982)	Microbiological Assay	-	Chloroform	2M HCl			1 ug/L (2 ml plasma)
Edstein (1984)	HPLC	UV detection 254 nm	ethylene chloride	phosphate buffer (pH 3.4)	10 um Bondapak C ₁₈	MeOH:CH ₃ CN:H ₂ O (25:15:60)	50 ug/L (0.5 ml plasma)

Cont. Table 5

Midskov (1984)	HPLC	UV detection 280 nm	Diethyl ether and Dichloromethane	Borate Buffer (pH 10)	5 um Spherisorb S5	<ol style="list-style-type: none"> 1. CH₃CN: Phosphate buffer (50:50) (pH 4, 0.1 M) 2. CH₃CN: phosphate buffer (pH 5.4, 0.1M) (30:70 & 20:80) 	Sufficiently sensitive
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- The extraction was repeated with another 6 ml aliquot of the extraction solvent.
- To enhance the recovery of sulfadoxine in the organic phase, 100 ul of isoamyl alcohol was added.
- The mixture was evaporated to dryness under a gentle stream of nitrogen at 60°C.
- The dried residue was reconstituted with 100 ul of methanol, and 20 ul was injected into the HPLC operated under the following conditions:

Detector: Ultraviolet detector set at 230 nm.

Column: Reverse phase C_{18} , (Lichrosorb RP-18), with packing material of particle size 5 μ m, and dimensions 125 x 4 mm.

Mobile Phase: Methanol:1% acetic acid, (27:73), at pH 3.0.

Flow rate of mobile phase: 1.5 ml/min.

Injection volume: 20 μ l

AUFS (detector): 0.1

Attenuation (integrator): 5

Chart speed: 0.2 cm/min

Extraction solvents reported for sulfadoxine were chloroform, (Weidekamm et al., 1982), dichloroethane, (Edstein, 1984 and Bonini et al., 1981), and dichloromethane, (Midskov, 1984). However, experiments conducted in our laboratory found that a mixture of diethylether and ethyl acetate, (1:1), gave the best recoveries with the most consistent reproducibility. In addition, the extraction solvent mixture has low boiling point, and evaporation could be achieved without heating at a high temperature. A 0.1M phosphate buffer, (pH 3.4), was used to extract sulfadoxine from plasma. This pH is similar to that used by Edstein, (1984), but with a different concentration of phosphate buffer. A 0.1M concentration gave better reproducibility of the extraction procedure. Figure 5.1 shows the chromatograms of sulfadoxine.

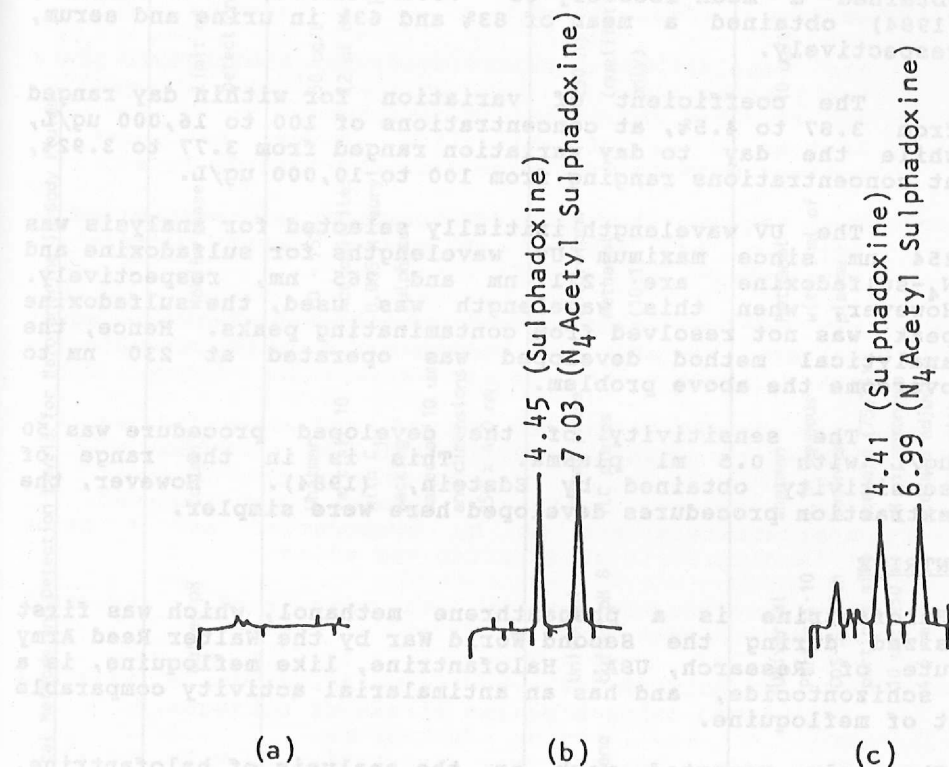


Figure 5.1: The chromatogram of Sulphadoxine analysis. (a) Blank plasma extract. (b) Methanolic standard containing 1 μ g Sulphadoxine and 1 μ g N_4 -Acetyl Sulphadoxine. (c) The plasma extract containing 1 μ g Sulphadoxine and 1 μ g N_4 -Acetyl Sulphadoxine.

The percent recovery of sulfadoxine was, however, rather poor with a mean recovery of $85.1 \pm 0.76\%$. This may be due to the poor solubility of sulfadoxine in organic solvents. The addition of isoamyl alcohol did help to improved the recovery somewhat. Relatively low recoveries have also been reported by other workers. Edstein, (1984), obtained a mean recovery of $78.6 \pm 13.5\%$, while Midskov, (1984) obtained a mean of 83% and 63% in urine and serum, respectively.

The coefficient of variation for within day ranged from 3.87 to 4.5%, at concentrations of 100 to 16,000 ug/L, while the day to day variation ranged from 3.77 to 3.92%, at concentrations ranging from 100 to 10,000 ug/L.

The UV wavelength initially selected for analysis was 254 nm since maximum UV wavelengths for sulfadoxine and N_4 -sulfadoxine are 271 nm and 265 nm, respectively. However, when this wavelength was used, the sulfadoxine peak was not resolved from contaminating peaks. Hence, the analytical method developed was operated at 230 nm to overcome the above problem.

The sensitivity of the developed procedure was 50 ug/L with 0.5 ml plasma. This is in the range of sensitivity obtained by Edstein, (1984). However, the extraction procedures developed here were simpler.

HALOFANTRINE

Halofantrine is a phenanthrene methanol, which was first synthesized during the Second World War by the Walter Reed Army Institute of Research, USA. Halofantrine, like mefloquine, is a blood schizonticide, and has an antimalarial activity comparable to that of mefloquine.

The only reported work on the analysis of halofantrine, other than that developed at our centre, was by Hines et al., (1985), see Table 6. The analytical method by Hines et al., (1985), is a reversed-phase HPLC method using paired-ion reagents, and ultraviolet detection at 254 nm. The chromatographic system consists of a Waters model M6000A single pump, a Waters fixed wavelength UV absorbance detector, set at 254, and a Waters U6-K manual injector, or a Waters Model 7108 autosample. The column used was a Whatman Partisil 10 column, packed with C_{18} bonded silica of particle size 10 μ m, with of dimensions 250 X 4.0 mm. The analytical column was maintained at $40.5 \pm 0.1^\circ\text{C}$.

The mobile phase used consisted of 70 - 75% acetonitrile and the corresponding percentage of water containing 0.005 M lauryl sulfate. The pH of the mobile was 3.5. This was achieved by using 10% (v/v) of sulfuric acid.

Table 6: Summary of Analytical Methods and Detection Levels for Halofantrine in Body Fluids

Author	Method of Analysis	Extraction Solvent	Buffer pH	Column	Mobile phase	Limit of Detection
Hines et al. (1985)	HPLC reverse phase with UV detector at 254 nm	30% ethyl acetate: hexane		Whatman Partisil 10 with C_{18} packing material 10 μ m and dimensions 250 x 4.0 nm	70 - 75% Acetonitrile: 0.005 M lauryl sulphate	50 ug/L (2 ml of plasma)
Kasipillai et al. (submitted for publication)	TLC	Hexane-dichloromethane (5:4)	Universal buffer pH 8	Normal phase TLC plates	Chloroform-methanol (10:1)	250 ng (qualitative only)
Kasipillai et al. (submitted for publication)	HPLC with UV detector at 254 nm	Hexane-buffer pH 8 (5:4)	Universal Partisil-10 ODS-3, with particle size 10 μ m; 250 x 4.6 nm	Whatman 0.1M aqueous ammonium acetate (75:25) pH of ammonium acetate adjusted to pH 4 with acetic acid	Methanol with 1 ml of plasma	10 ug/L

The extraction procedure was as follows:

- To two ml aliquots of blood were added 500 - 800 ug/L of internal standard buffer, and 4 ml of 30% ethylacetate:hexane.
- The samples were extracted overnight by gentle shaking.
- The samples were centrifuged and the organic phase was removed.
- Another 2 ml of extraction solvent was used to extract more halofantrine from the aqueous layer.
- The organic layers were pooled and evaporated to dryness by passing through a gentle stream of nitrogen.
- The dried residue was partitioned between equal volume, (4 ml), of hexane and 0.1M citric acid (in 90% H₂O:MeOH).
- The hexane layer was removed, and discarded, and the procedure repeated with another 4 ml of hexane.
- The aqueous methanol phase was reduced to approximately 0.5 ml by evaporation and 4 ml of trisodium:boric acid buffer was added.
- The buffer was partitioned with 4 ml of 30% ethyl acetate:hexane.
- The organic layer was transferred to a vial and evaporated using a gentle stream of nitrogen.
- The dried residue was reconstituted with 200 ul of acetonitrile: methanol (1:1) and injected into the HPLC.

The above extraction procedure was rather tedious and time consuming for routine work, so a simpler extraction procedure was necessary. The mean recovery from whole blood was 84%, with a CV of 5%, at concentrations ranging from 50 - 1000 ug/L. The detection limit for halofantrine obtained was approximately 10 ug/L with 2 ml of whole blood.

Due to the unavailability of sensitive analytical methods for halofantrine in body fluids, very little is known about the metabolism and pharmacokinetics of halofantrine in man. The absorption of halofantrine from currently available oral formulations varies unpredictably, and more work to gain more information on the pharmacokinetic behavior of halofantrine is needed.

As a result of the above need, a sensitive, and specific, analytical method was developed by Kasipillai et al. at the Centre for Drug Research, Universiti Sains Malaysia, Penang.

TLC Method For Halofantrine

Plasma extracts of halofantrine were chromatographed on normal phase TLC plates, with chloroform-methanol, (10:1), as the solvent system. The following methods or reagents were used to detect the drug:

- (a) exposure to ultraviolet light at 254 nm;
- (b) exposure to iodine vapour;
- (c) sprayed with iodoplatinate acid reagent; or
- (d) sprayed with Dragendorff reagent.

The R_F values were calculated. The lowest detection limit for halofantrine was obtained qualitatively with reagents (c) and (d) respectively.

The detection limit for halofantrine, using the TLC method, is 250 ng (qualitatively), which is not sensitive enough for pharmacokinetic studies but may be used for screening purposes.

HPLC Method for Halofantrine

The use of the reverse-phase HPLC method developed here enabled halofantrine to be detected, at a level as low as 10 ug/L of plasma, using an ultraviolet detector set at wavelength 254 nm. The extraction method was less tedious than that developed by Hines et al. (1985), and is more selective, sensitive and reproducible.

The chromatographic system consisted of a double pump, (Waters Associate Pump Model 501), a fixed wavelength UV absorbance detector, (Waters Associate Model 440), set at 254 nm, and a manual injector, (Rheodyne Model 7125). Chromatographic separation was performed with a Whatman Partisil-10 ODS-3 reverse phase column, with packing material of particle size 10 um and dimensions of 250 X 4.6 mm. The mobile phase comprised of 75% methanol and 25% water containing 0.1M ammonium acetate. The pH of the ammonium acetate solution was adjusted to pH 4 with acetic acid.

The extraction procedure was performed as follows:- to 1 ml of plasma was added 1 ml of universal buffer, (pH 8), and 250 ul saturated sodium sulphate was extraction

with 5 ml of hexane-dichloromethane (5:4). The samples were then centrifuged at 2500 rpm for 10 minutes. The organic layer was transferred to a clean test-tube. The aqueous layer was rinsed further with 2 ml of the extraction solvent and the organic layer separated and combined, with the above organic phase. The sample extracts were evaporated to dryness under a gentle stream of nitrogen at room temperature. The dried residue was reconstituted with 100 μ l of methanol and 20 μ l injected into the HPLC.

Figure 6.1 shows the chromatograms of plasma samples of halofantrine.

Calibration curves for halofantrine were linear in the range of 10 - 1000 μ g/L of plasma ($r^2 = 1.0$). The sensitivity limit of the assay is 10 μ g/L plasma. In plasma samples, the within day variation of halofantrine ranged from 1.21 to 5.48% for concentrations ranging from 20 to 200 μ g/L. The day to day variation of halofantrine ranged from 1.06 to 7.73% for concentration ranging from 25 to 200 μ g/L. The mean recovery of halofantrine from plasma is $89.91 \pm 2.86\%$.

The method developed here has been used to study the pharmacokinetics of halofantrine, in man, after a single dose of 250, 500 and 1000 mg halofantrine, respectively.

PYRONARIDINE

Pyronaridine is a new antimalarial, and is being studied to determine the chemotherapeutic and toxic effects in animals. The New Drug Group of the former Department of Malaria, Institute of Parasitic Diseases, Shanghai, (1980), found that pyronaridine was effective against chloroquine-resistant *Plasmodium falciparum* and cerebral forms of malaria.

Due to the lack of a sensitive analytical method, no previous work had been done to determine the concentration of pyronaridine in plasma, or whole blood. A sensitive analytical method was recently developed by the Centre for Drug Research, Universiti Sains Malaysia, Penang recently. HPLC method with ultraviolet detection at 278 nm. The HPLC chromatographic system consists of a dual Gilson Model 330 pump and a Rheodyne 7125 injector, with a Waters Lambda-Max Model 481 ultraviolet detector set at wavelength 278 nm. A guard column of dimensions 3 cm x 4 mm i.d., and packed with C_{18} packing material of particle 10 μ m, was attached to the analytical column. The analytical column was a Whatman Partisil-10 ODS column of dimensions 25 cm x 4.6 mm i.d., and C_{18} packing material of 10 μ m. The mobile phase consisted of 1% triethylamine and 0.08M potassium dihydrogen phosphate buffer: acetonitrile, (85:15, v/v). The pH of the aqueous buffer, in the mobile phase, was maintained at 2.80 with ortho-phosphoric acid. The mobile phase flow rate was maintained at 1.5 ml/min.

The extraction procedure for pyronaridine from whole blood and plasma were carried out as follows:

To 100 μ l of plasma was added 250 μ l of chloroform (internal standard), and 500 μ l of chloroform. The mixture was vortexed for 2 minutes, and centrifuged at 2500 rpm for 10 minutes. The organic layer was removed and dried under nitrogen. The dried residue was reconstituted in 100 μ l of methanol and 20 μ l injected into the HPLC. Recovery of pyronaridine from plasma and plasma was good, with a mean recovery of 89.91% at concentrations ranging from 10 to 1000 μ g/L. The mean within-day variation of pyronaridine from plasma was 1.21% at concentrations ranging from 20 to 200 μ g/L. While the mean day to day variation was 5.48%, at concentrations ranging from 25 to 200 μ g/L.

For whole blood, the mean within day variation of pyronaridine was 5.07%, at concentrations ranging from 10 to 1000 μ g/L. While the mean day to day variation was 7.73%, at concentrations ranging from 25 to 200 μ g/L. The mean within-day variation of pyronaridine in plasma was 1.06% at concentrations ranging from 25 to 200 μ g/L. The day to day variation of pyronaridine in plasma was 7.73% at concentrations ranging from 25 to 200 μ g/L.

Figure 6.1: Chromatograms for the halofantrine analysis

(a) Standard halofantrine sample (On column concentration, 100 ng)
 (b) Blank plasma (interference peak, RT = 10.33 min)
 (c) Spiked plasma (500 ng/ml)
 (d) Plasma sample at 0 hr
 (e) Plasma sample, 1 hr after drug administration
 (f) Plasma sample, 6 hr after drug administration

mobile phase, 75% MeOH - 0.1M CH_3COONH_4 (pH 4);
 Flow rate, 1.5 ml/min

The extraction procedure for pyronaridine from whole blood and plasma were carried out as follows:

- To 200 μ l of plasma was added 250 ng chloroquine diphosphate, (internal standard), and 500 μ l trizma buffer.
- Three ml of diethylether was used to extract the compounds of interest.
- Following vortexing for 2 minutes, and centrifugation at 2,500 rpm for 10 min, the organic layer was removed and dried under nitrogen gas.
- The dried residue was then reconstituted in 100 μ l mobile phase and 50 μ l was injected into the HPLC.

Recovery of pyronaridine from plasma and plasma was good, with a mean percent recovery of %, at concentrations ranging from 70 to 5,000 μ g/L. The mean within-day variation of pyronaridine from plasma was 5.39%, at concentrations ranging from 70 to 5,000 μ g/L, while the mean day to day variation was 3.83%, at concentrations ranging from 125 to 2,000 μ g/L.

For whole blood, the mean within day variation of pyronaridine was 5.07%, at concentrations ranging from 70 to 4,000 μ g/L, while the mean day to day variation was 4.44%, at concentrations ranging from 125 to 2,000 μ g/L.

The minimum detectable concentration of pyronaridine is 14 μ g/L, with 1 ml of plasma or whole blood.

Figure 7.1 shows the chromatogram of pyronaridine in plasma samples.

PROGUANIL AND THE OTHER BIGUANIDES

Reports of the continuing prophylactic activity of proguanil, in areas affected by chloroquine-resistant and pyrimethamine-resistant *P. falciparum*, (McLarthy et al., 1984), have prompted new interest in this well tolerated drug. However, from these reports it appears that a higher daily dose of proguanil is required than that originally recommended. A review of the literature revealed a paucity of reports on the pharmacokinetics of proguanil and cycloguanil. This was largely due to inadequately sensitive analytical methods. The available analytical methods are listed in Table 7. The detection levels obtained ranged from 10 to 60 μ g/L.

To determine proguanil and cycloguanil in the human plasma, a high performance liquid chromatograph, (HPLC), with ultra-violet, (UV), detection, was developed in our laboratory. A solid phase extraction, (SPE), procedure was developed as follows:

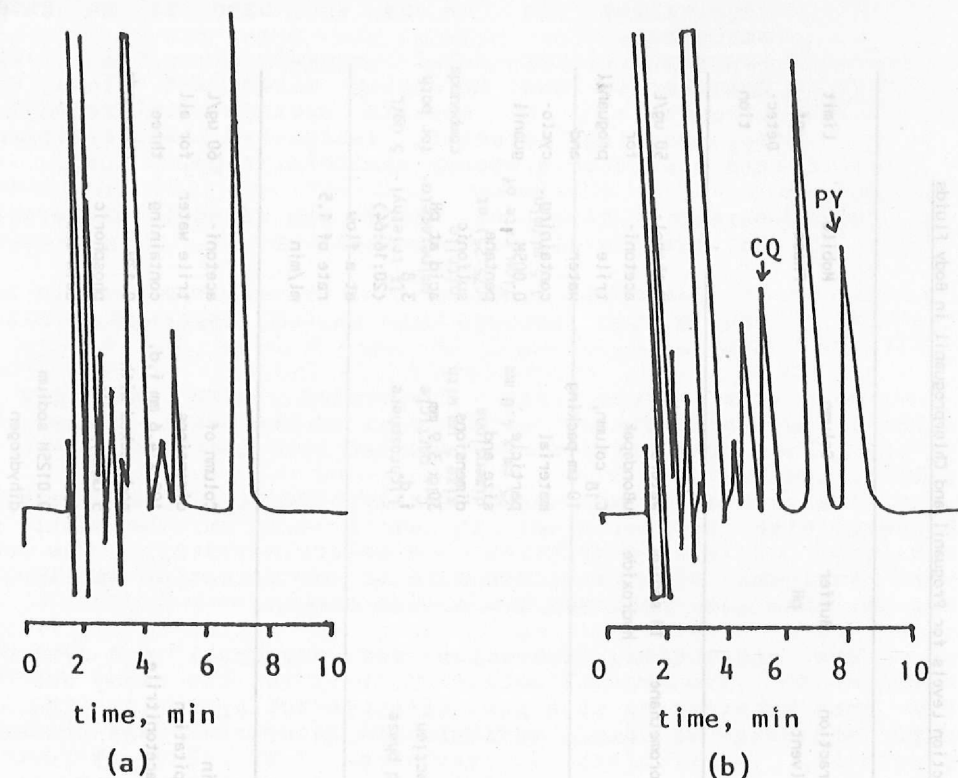


Figure 7.1: Chromatogram for (a) blank plasma and (b) plasma extract of 100 ng pyronaridine tetrathosphate and 250 ng chloroquine diphosphate
CQ = chloroquine (Retention time = 5.82 min)
PY = pyronaridine (Retention time = 8.80 min)

Table 7: Summary of Analytical Methods of Detection Levels for Proguanil and Chlorproguanil in Body Fluids

Compound	Author	Method of Analysis	Extraction Solvent	Buffer pH	Column	Mobile Phase	Limit of Detection
Proguanil and Cyclo-	Edstein (1986)	HPLC reversed phase with UV detection at 238 nm	dichloromethane	1M sodium hydroxide	Waters uBondapak C ₁₈ column, 10 um packing material particle size and dimensions 30 x 3.9 mm i.d.	Methanol-acetonitrile water containing 0.005M pentane sulfonic acid at pH 3.8 (20:16:64) at a flow rate of 1.5 ml/min	50 ug/L for proguanil and cyclo-guanil
Proguanil cycloguanil and 4-chloro-phenylbiguanide	Moody et al. (1980)	HPLC method with ultraviolet detection at 247 nm	protein precipitation with acetonitrile		Column of dimensions 100 x 4.6 mm i.d. and packed with 5 um Hypersil ODS 0.0125M sodium dihydrogen phosphate (50:50)	acetonitrile water for all containing 0.17M phosphoric acid and	60 ug/L for all three compounds

Cont. Table 7

Proguanil and cycloguanil	A. Jamaludin et al. (1989) submitted for publication	HPLC reversed-phase with UV detection at 248 nm.	Solid phase extraction		Lichrosphere C ₁₈ particle size 5 um with dimensions 25 cm x 4.0 mm	1% triethylamine:acetonitrile (75:25) at flow rate of 1.5 ml/min.	5 ug/L for both compounds
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- One ml of plasma was mixed with one ml 0.1M ammonium acetate, and 40 ul pyrimethamine solution, (equivalent to 80 ng and used as an internal standard).
- The sample was thoroughly mixed and slowly eluted through a 1 ml insulin syringe containing 100 mg C₁₈ packing material, (which had been conditioned), and placed on a vacuum manifold (Supelco Inc.).
- Three X 1 ml of water was added into the column, followed by 125 ul acetonitrile.
- On complete elution, 1 ml of ammonium acetate buffer was eluted through the column, followed by 3 X 1 ml methanol.
- The elute was evaporated with nitrogen and reconstituted with 100 ul of the mobile phase. 50 ul of the extract was injected into the HPLC.

The column used was a Lichrosphere C₁₈, (particle size of 5 um), with dimension of 25 cm X 4.0 mm; the mobile phase consisted of 1% triethylamine: acetonitrile (75:25). The samples were analysed with a Gilson HPLC at the detection wavelength of 248 nm. The flow rate was 1.5 ml per minute.

The extraction recoveries for proguanil and cycloguanil were > 96%. The minimal detection limit for the assay was 5 ug/L for both substances, at a peak to noise ratio of 3:1. The within day, and day to day, variation in recoveries were greater for proguanil, (coefficient of variation 7.2% at 95.7% recovery), than for cycloguanil, (coefficient of variation 4.5% at 97.3% recovery).

REFERENCES

Chloroquine

- Adelusi, S.A. and Salako, L.A., (1980): Improved Fluorimetric Assay of Chloroquine in Biological Samples. Journal of Pharmacy and Pharmacology, 32, 711.
- Alvan, G., Ekman, L. and Lindstrom, B. (1982): Determination of Chloroquine and its Desethyl Metabolite in Plasma, Red Blood cells and Urine by Liquid Chromatography. Journal of Chromatography, 229, 241-247.
- Bergqvist, Y. and Frisk-Holmberg, M. (1980): Sensitive Method For the Determination of Chloroquine and its Metabolite Desethyl-Chloroquine in Human Plasma and Urine by High-Performance Liquid Chromatography. Journal of Chromatography, 221, 119-127.
- Brodie, B.B., Udenfriend, S., Dill, W. and Chenkin, T. (1947): The Estimation of Basic Organic Compounds in Biological Material. III. Estimation by Conversion to Fluorescent Compounds. Journal of Biological Chemistry, 168, 319-325.
- Kuye, J.O., Wilson, M.J. and Walle, T. (1983): Gas Chromatographic Analysis of Chloroquine After a Unique Reaction with Chloroformates. Journal of Chromatography, 272, 307-313.
- McChesney, E.W., Wyzan, H.S. and McAuliff, J.P. (1956): The Determination of 4-aminoquinoline antimalarials: Revaluation of the Induced Fluorescence Methods, With Specific Application to Hydroxychloroquine Analysis. Journal of Pharmaceutical Sciences, 45, 640-645.
- Mihaly, G.W., Nichols, D.D., Edwards, G., Ward, S.A., Orme, M.L.E., Wasrell, D.A. and Breckenridge, A.M. (1985): High-performance Liquid Chromatographic Analysis of Amodiaquine in Human Plasma. Journal of Chromatography, 337, 166-171.
- Pussard, E., Verdier, F. and Blayo, M.C. (1986): Simultaneous Determination of Chloroquine, Amodiaquine and their Metabolites in Human Plasma, Red Blood Cells, Whole Blood and Urine by Column Liquid Chromatography. Journal of Chromatography, 374, 111-118.
- Williams, S.B., Patchen L.C. and Churchill, F.C. (1988): Analysis of Blood and Urine Samples for Hydroxychloroquine and Three Major Metabolites by High-performance Liquid Chromatography with Fluorescence Detection. Journal of Chromatography, 433, 197-206.

Quinine and Quinidine

- Arunyanart, M. and Cline Love, L.J. (1985): Determination of Drugs in Untreated Body Fluids by Micellar Chromatography with Fluorescence Detection. Journal of Chromatography, 342, 293-301.
- Barrows, S.E., Taylor, A.A., Horning E.C. and Horning, M.G. (1980): High-performance Liquid Chromatographic Separation and Isolation of Quinidine and Quinine Metabolites in Rat Urine. Journal of Chromatography, 181, 219-226.
- Cramer, G. and Isaksson, B. (1963): Quantitative Determination of Quinidine in Plasma. Scandinavian Journal of Clinical Laboratory Investigations, 15, 553.
- Edstein, M., Stace, J. and Sharm, F. (1983): Quantification of Quinine in Human Serum by High-performance Liquid Chromatography. Journal of Chromatography, 278, 445-451.
- Furner, R.L., Brown, G.B. and Scott, J.W. (1981): A Method for Differentiation and Analysis of Quinine and Quinidine by Gas Chromatography/Mass Spectrometry. Journal of Analytic Toxicology, 5, 275-278.
- Guentert, T.W., Rakhit, A., Upton, R.A. and Riegelman, S. (1980): An Integrated Approach to Measurements of Quinidine and Metabolite in Biological Fluids. Journal of Chromatography, 183, 514-518.
- Leroyer, R., Jarreau, C. and Pays, M. (1982): Specific Determination of Quinidine and Metabolites in Biological Fluids by Reversed-phase High-performance Liquid Chromatography. Journal of Chromatography, 228, 366-371.
- Mihaly, G.W., Hyman, K.M., Smallwood, R.A. and Hardy, K.J. (1987): High-performance Liquid Chromatographic Analysis of Quinine and its Diastereoisome Quinidine. Journal of Chromatography, 415, 177-182.
- Ochs, H.R., Greenblatt, D.J. and Woo, E. (1980): Clinical Pharmacokinetics of Quinidine. Clinical Pharmacokinetics, 5, 150-168.
- Reece, P.A. and Peikert, M. (1980): Simple and Selective High-Performance Liquid Chromatographic Method For Estimating Plasma Quinidine Levels. Journal of Chromatography, 181, 207-217.
- Spinks, A. and Tottey, M.M. (1948): Studies on Synthetic Antimalarial Drugs. XVI. The Absorption, Distribution and Excretion of 2-p-chlorophenylguanidino-4-diethylaminoethyl-amino-6-methylpyrimidine (3349) in Experimental Animals. Annals of Tropical Medicine and Parasitology, 40, 145-152.

Mefloquine

- Dadgar, D., Climax, J., Lambe, R. and Darragh, A. (1985): Gas Chromatographic Determination of Mefloquine in Human and Drug Plasma Using Electron-capture Detection. Journal of Chromatography, 337, 47-54.
- Desjardins, R.E., Pamplin, C.L., Von Bredow, J., Barry, K.G. and Canfield, C.J. (1979): Kinetics of a New Antimalarial, Mefloquine. Clinical Pharmacology and Therapeutics, 26, 372-379.
- Grindel, J.M., Tilton, P.F. and Shafler, R.D. (1977): Quantitation of the Antimalarial Agent Mefloquine in Blood, Plasma and Urine Using High-Performance Liquid Chromatography. Journal of Pharmaceutical Sciences, 66, 834-837.
- Hezmann, P. and Geschke, R. (1984): Determination of The Antimalarial Mefloquine in Human Plasma by Gas Chromatography with Election-Capture Detection. Journal of Chromatography, 311, 411-417.
- Kapetonovic, I.M., Digioranni, J.D., Bartosevich, J., Melendez, V., Von Bredow, J. and Heiffer, M. (1983): Analysis of the Antimalarial, Mefloquine, in Blood and Plasma Using High-Performance Liquid Chromatography. Journal of Chromatography, 277, 209-215.
- Nakagawa, T., Higuchi, T., Haslam, J.L., Shaffer, R.D. and Mendenhall, D.W. (1979): Gas Liquid Chromatography Determination of Whole Blood Antimalarial Concentration. Journal of Pharmaceutical Science, 68(6).
- Schwartz, D.E., Weber, B., Richard-Lenoble, D., Gentilini, M. (1980): Kinetic Studies of Mefloquine and one of its Metabolites, RO 21-5104, in the Dog and in Man. Acta Tropica, 37, 238-242.
- Schwartz, D.E. and Renalder, U.B. (1981): Highly Sensitive and Specific Determination of Mefloquine in Biological Fluids Using Gas Chromatography Mass Spectrometry with Selected Ion-Monitoring. Journal of Biomedical Mass Spectrometry, 8(12), 589-592.

Pyrimethamine

Ahmad, R.A. and Rogers, H.J. (1980): Pharmacokinetics and Protein Binding Interactions of Dapsone and Pyrimethamine. British Journal of Clinical Pharmacology, 10, 519-524.

Bonini, M., Mohofio, F. and Barazi, S. (1981): Contribution on Dosage of 4 Antimalarial Agents, Chloroquine, Quinine, Pyrimethamine and Sulphadoxine, Alone and Combined in Biological Milieu. 2. Gas-phase Chromatograph. Journal of Chromatography, 224 (2):332-337.

DeAngelis, R.L., Simmons, W.S. and Nichols, C.A. (1975): Quantitative Thin-Layer Chromatography of Pyrimethamine and Related Diaminopyrimidines in Body Fluids and Tissues. Journal of Chromatography, 106, 41-49.

Edstein, M. (1984): Simultaneous Measurement of Sulphadoxine, N₄-Acetylsulphadoxine and Pyrimethamine in Human Plasma. Journal of Chromatography, 305, 502-507.

Jones, C.R. and Overell, S.M. (1979): Determination of Plasma Concentrations of Dapsone, Monoacetyl Dapsone and Pyrimethamine in Human Subjects Dosed with Maloprim. Journal of Chromatography, 163, 179-185.

Jones, C.R., Ryle, P.R. and Weatherly, B.C. (1981): Measurement of Pyrimethamine in Human Plasma by Gas-liquid Chromatography. Journal of Chromatography, 224, 492-495.

Levin, E.M., Meyer, R.B. and Levin, V.A. (1978): Quantitative High Pressure Liquid Chromatographic Procedure for the Determination of Plasma and Tissue Levels of 2,4-diamino-5-(3,4-dichloro phenyl)-6-methylpyrimidine (Metoprime) and its Application to the Measurement of Brain Capillary Permeability Coefficients. Journal of Chromatography, 156, 181-187.

Martindale (1982): The Extra Pharmacopoeia 28th Edition, J.E.F. Reynolds (Ed). The Pharmaceutical Press, London.

Midskov, C. (1984a): High Performance Liquid Chromatographic Assay of Pyrimethamine, Sulphadoxine and its N⁴-Acetyl Metabolite in Serum and Urine After Ingestion of Suldox. Journal of Chromatography, 306, 388-393.

Midskov, C. (1984b): Rapid Gas Chromatographic Determination of Pyrimethamine in Human Plasma and Urine. Journal of Chromatography, 308, 217-227.

Simmons, W.S. and DeAngelis, R.L. (1983): Quantitation of Pyrimethamine and Related Diaminopyrimidine in Situ by Enhancement of Fluorescence After Thin-Layer Chromatography. Journal of Analytical Chemistry, 45(8), 1538-1541.

Smith, R.V. and Stewart, J.T.: Textbook of Biopharmaceutic Analysis, Lea and Febiger Publishers, Philadelphia, USA.

Timm, U. and Weidekamm, E. (1982): Determination of Pyrimethamine in Human Plasma After Administration of Fansidar or Fansidar-Mefloquine by Means of High Performance Liquid Chromatography with Fluorescence Detection. Journal of Chromatography, 230, 107-114.

Weidekamm, E., Plozza-Nottebrock, H., Forgo, J. and Duback, U.S. (1982): Plasma Concentrations of Pyrimethamine and Sulphadoxine and Evaluation of Pharmacokinetics Data by Computerised Curve-Fitting. Bulletin of the World Health Organization, 60(1), 115-122.

Sulfadoxine

Bonini, M., Mohofio, F. and Barazi, S. (1981): Contribution on dosage of 4 antimalarial agents, chloroquine, quinine, pyrimethamine and sulphadoxine, alone and combined in biological milieu. 2. Gas-phase chromatograph. Journal of Chromatography, 224(2): 332-337.

Bratton, A.C. and Marshall, E.K. (1939): A New Coupling Component for Sulphanilamide Determination. Journal of Biological Chemistry, 128, 537-550.

Clarke, E.G.C. (1978): Isolation and Identification of Drugs in Pharmaceuticals, Body Fluids and Post-mortem Material, Berle, Vol 1 and 2, The Pharmaceutical Press, London.

Edstein, M. (1984): Simultaneous Measurement of Sulphadoxine, N₄-Acetylsulphadoxine and Pyrimethamine in Human Plasma. Journal of Chromatography, 305, 502-507.

Fagerlund, C. and Hartvig, P. (1979): Extractive Alkylation of Sulphonamide Diuretics and Their Determination by Electron-Capture Gas Chromatography. Journal of Chromatography, 168, 107.

Gyllenhaal, O., Naslund, B. and Hartvig, P. (1978): Electron-capture Gas Chromatography of Sulphadoxine and its N⁴-Acetyl Metabolite in Serum After Extractive Methylation. Journal of Chromatography, 156, 330-334.

Helboe, P. and Thomsen, M. (1977): High Performance Liquid Chromatographic Determination of Trimethoprim and Sulphonamide Combinations in Pharmaceuticals. Archives of Pharmacy and Chemical Science, 5, 25-32.

Midskov, C. (1984): High Performance Liquid Chromatographic Assay of Pyrimethamine, Sulphadoxine and its N⁴-Acetyl Metabolite in Serum and Urine Ingestion of Suldox. Journal of Chromatography, 308, 217-227.

Weidekamm, E., Plozza-Nottebock, H., Forgo, J. and Duback, U.S. (1982): Plasma Concentrations of Pyrimethamine and Sulphadoxine and Evaluation of Pharmacokinetics Data by Computerised Curve-Fitting. Bulletin of the World Health Organization, 60(1), 115-122.

Halofantrine

J.W. Hines, P.D. Elkins, C.E. Cook and C.M. Sparacino, (1985): Paired-Ion Liquid Chromatographic Method for the Analysis of a Phenanthrenemethanol antimalarial in whole blood. Journal of Pharmaceutical Sciences, 74(4), 433-437.

Kasipillai C., V. Navaratnam and A. Jamaludin (1989): Development of a Reliable and Sensitive Assay Procedure for Halofantrine Hydrochloride. (Submitted for Publication).

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Proguanil

Edstein, M.D. (1986): Simultaneous Measurement of Proguanil and Cycloguanil in Human Plasma by High-performance Liquid Chromatography. Journal of Chromatography, 380: 184-189.

Jamaludin, A., Mohamed, M., Nair, N.K. and Navaratnam, V. (Submitted for Publication).

McLarty, D.G. Webber, R.H., Jaatinen, M., Kihamia, C.H., Murru, M., Kumano, M. Auber, B. & Magnuson, L.W. (1984): Chemoprophylaxis of Malaria in Non-immune Residents of Dar Es Salaam, Tanzania, Lancet, ii: 656-659.

Moody, R.R., Selkirk, A.B. & Taylor, R.B. (1980): High-performance Liquid Chromatography of Proguanil, Cycloguanil and 4-chlorophenyl-biguanide Using Hydrophobic Pairing Ion and its Application to Serum Assay. Journal of Chromatography, 182: 359-367.

REVIEW OF DATA DERIVED FROM FIELD TESTS FOR CHLOROQUINE CONDUCTED IN SRI LANKA AS FOLLOW-UP STUDIES TO THE WORKSHOP

Krisantha Weerasuriya

Introduction

Two field tests are being evaluated. The first is the Saker-Solomons modification I (Mount et al., 1989) and the other is the ELISA for chloroquine developed by a group in Sunderland, U.K. (Rowell et al., 1988).

Saker-Solomons Modification I (SS/CQ1)

Two studies were undertaken.

Aim

1. To follow the pattern of excretion in the urine of chloroquine, and metabolite, after a single dose of 300mg of chloroquine base (Volunteer Study).
2. Screening of urine for chloroquine in patients suspected of malaria, (Patient Study).

Method

Volunteer Study

Six healthy male volunteers between 27-50 years, whose urine was negative for SS/CQ1, were given two tablets of chloroquine (=300 mg base). These subjects had not taken any chloroquine in the past four months; two subjects had taken two tablets for a study before that, but none of the volunteers had taken chloroquine for malaria within the past five years. Urine samples, obtained in the morning, were tested for chloroquine with SS/CQ1 on the same day. Where testing on the same day was not possible, the urine was stored at 4°C. The maximum time between voiding and testing was 48 hours. On day 8, (day 0 = tablets administered), urine and blood samples were taken for assay of chloroquine and desethylchloroquine. The SS/CQ1 was done until the test was negative on three consecutive days. Permission for the study was obtained from the ethical committee of the Faculty of Medicine, University of Colombo, Colombo.

Patient Study

On request, the Department of Parasitology of the Medical Faculty, screens blood films of patients, in the General Hospital, Colombo for the malaria parasite. These patients are generally residents of Colombo or its suburbs. Malaria transmission does not occur in these areas, and infection generally occurs during brief visits to areas where malaria is

endemic. Some patients were recruited from the headquarters of the Anti Malaria Campaign, (AMC), in Colombo. Although this is an administrative centre, a small de facto outpatient clinic operates for patients who suspect they have malaria and are seeking treatment at the headquarters of the Campaign.

At the time of blood filming, a urine sample was obtained and a detailed questionnaire, which included a drug history, was filled in by a medical student. The two services operate on week days; the urine collected was tested on the same day.

The first study was finished in late July. The second was begun in late July and is still continuing; about 130 patients have been screened so far.

Results

Volunteer Study

Urine of all the 6 subjects tested were positive by SS/CQI up to day 14. On day 15, one subject became negative and remained so, while another defaulted in attendance. The third subject became negative on day 16 and remained negative. The last three subjects became negative after day 18, but subsequently had intermittent positives. The last positive was seen on day 22.

The sum of the plasma values for chloroquine and desethylchloroquine, (assayed by HPLC), on day 8 ranged from 25-60 ug/L, and the urine values from 1500-3550 ug/L. Further analysis of samples on the HPLC was not done due to problems with the HPLC machine.

Patient Study

One hundred and thirty four urine samples were tested by SS/CQI. Of these 121 were from patients of General Hospital, Colombo and the rest from AMC.

Seventy on samples were positive by the SS/CQI. Of these 35 had a history of recent chloroquine ingestion, as assessed by history or patient records. These samples were taken as positive for chloroquine.

Of the 36 who were positive for SSCQI but had no history of chloroquine ingestion, 8 were found to be positive and 15 negative for CQ by ELISA; the rest, (12), are still awaiting analysis.

Of the remaining 27 urines, (15 false positives and 12 awaiting testing), 15 subjects had taken drugs capable of giving a false positive. Chlorpheniramine, promethazine and ephedrine was commonly recorded and, thus, all three were capable of producing false positives: Ephedrine, even as nasal drops, was found to be produce a false positive.

To summarize, urines, from 122 patients suspected of having malaria, were tested for chloroquine by the SS/CQI test and, where a false positive was suspected, cross checked with ELISA. Fifteen true false positives, as judged by ELISA, have so far identified.

A further 12 samples are suspected to be false positive, but these await analysis with ELISA.

Discussion

Volunteer Study

The urine of all subjects was positive for chloroquine, as judged by SS/CQI, for a minimum of 14 days. The dose was 300mg which is the standard dose for the weekly prophylactic regime. Since chloroquine has a long half life, it would take more than one week to reach steady state on a weekly prophylactic regime. Hence, the excretion during the later weeks when a steady state is reached. Therefore, if the SS/CQI can detect chloroquine throughout the first week, it should be adequate to check on compliance during the entire period a subject is on prophylaxis. The HPLC values also confirm that amounts above 1 mg/L were excreted in the urine. The trough plasma values for the sum of chloroquine and desethylchloroquine were between 25-60 ug/L. Presumably, values around this level would be sufficient to protect a subject from malaria.

Patient Study

The sensitivity of the Saker-Solomons was satisfactory. All patients who would have been expected to be positive, (by recent ingestion of chloroquine on history or patient notes), had a positive urine sample. However, these positives were not confirmed with ELISA. Therefore, false positives cannot be excluded. But they are very unlikely.

The false positives seen decreases the value of the test. Antihistamines are commonly administered with chloroquine to prevent vomiting and would prevent the interpretation of a positive SSCQI. Phenothiazines and tricyclic antidepressants were known to give false positives in the SS/CQI. We have discovered that codeine and pilocarpine also give false positives. Morphine, paracetamol, adrenaline, histamine, (the last two were tested with spiked samples of urine), did not give false positives.

Other Observations

Some other observations were made during this study. After a full course of chloroquine, (25mg/kg), the urine could be expected to be positive for 6-8 weeks. This may have some value in evaluating whether a subject has taken a full course of

chloroquine within that period. However, another group in Sri Lanka, (Personal communication, Abeysekera), have found it to be positive only up to two weeks. This discrepancy is unexplained and further work is necessary.

The chloroquine in the urine appears stable. Keeping the urine samples at room temperature for 48 hours does not appear to affect the results with SSCQI. Urine stored at 4°C has a precipitate; SS/CQI was done from the clear urine and again there seemed to be no difference from the result done with the freshly voided sample. A more sensitive quantitative method would be required to ascertain whether minor amounts of deterioration will occur.

Ten urine samples of patients taken 24 hours after the first dose of chloroquine, (4 tablets = 600 mg of base) was checked with the SS/CQI. They all had more than 3 mg/L; however, this is unlikely to be a good test for absorption of chloroquine. More than 20 mg/L may be excreted at 24 hours, therefore, a marked decrease of absorption would have to occur for SS/CQI to be affected.

ELISA (Rowell et al., 1988)

The evaluation of this method was begun in early September. Our experience during these four weeks was limited to familiarizing ourselves with the limits of the assay, and using it to assess suspected false positives in the Saker-Solomon test.

Calibration

Calibration curves with 6 standards have been satisfactory with correlation coefficients over 0.95. In addition to the recommended PBST, we have evaluated blank urine as a diluent because ELISA could be used to measure urinary chloroquine. Blank urine as a diluent was inferior to the recommended PBST as a diluent. With experience, we have achieved incubation times that produce a adequate difference between the optical density readings of the highest and lowest standards.

Reagents

The reagents appear stable except the substrate for the enzyme, which deteriorates about 24 hours after reconstitution. A yellow colour is produced on deterioration providing a convenient marker for spoilt substrate.

Minimum Level of Detection - Sensitivity

Our calibration standards have ranged between 10,000-1 ug/L. At present, readings below 30 ug/L have too much variability. With further practice it might be possible to

achieve better results. Also, the exclusive use of PBST as the diluent would improve the reproducibility, as some of these tests were done with blank urine as a diluent.

Day to Day Variability

This has been disappointing as some of the urine samples used for "yesterday/today/tomorrow" have shown wide variation. The urine was stored at -20°C and samples for testing were taken after thawing from the clear supernatant. This may have some affect on the result and further evaluation is necessary.

Cross Reaction with the Metabolite - Desethylchloroquine

Solutions of the metabolite, with concentrations ranging from 1 to 100,000 ug/L, were tested. Except at the very highest levels, which would be unlikely to be seen in clinical practice, there were no positive readings. Contamination by chloroquine, at the highest concentration, cannot be excluded. ELISA, when assaying chloroquine at levels seen in clinical practice, appears to be specific to the side chain rather than quinoline nucleus.

Non Specific Bindings and Readings with Blank Urine

Readings above 2 are beyond the useful range of the microplate reader used in ELISA. The 25 blank urines tested have all given values above 2. Non-specific binding was assessed by omitting one reagent at a time from a series of assay runs using the same samples. There was a minimal colour change, but the optical density readings were completely outside the range of the calibration curve.

In summary, our brief experience has shown ELISA to have a good potential. Its specificity is very good; even the metabolite did not produce a cross reaction. The sensitivity is much better than the Saker-Solomons, but at present inferior to that of HPLC. However, it may be possible to achieve greater sensitivity with further experience of the technique. This improvement over the Saker-Solomons has required personnel with more training, expensive chemicals, and a longer time for each test. Whether this method can be successfully transferred from a university department to health care institutions, with more basic facilities, also needs to be evaluated.

Reference

Mount, D.L., Nahlen, B.L., Patchen, L.C. & Churchill, F.C. (1989). Adaptations of the Saker-Solomons test: simple reliable colorimetric field assays for chloroquine and its metabolite in urine. Bulletin of the World Health Organisation, 67, 295-300.

Rowell, V., Rowell, F.J., Baker, A., Laurie, D. & Sidki, A. M. (1988) A specific ELISA method for determining chloroquine in urine or dried blood spots. Bulletin of the World Health Organisation. 66, 211-217

FIELD ASSAYS OF ANTIMALARIAL DRUGS: WHAT IS THE NEED FOR IT IN TREATMENT AND RESEARCH IN MALARIA?

Krisantha Weerasuriya

Introduction

Self medication with antimalarial drugs, especially chloroquine, is a widespread and increasing phenomenon in countries with malaria. Chloroquine is also widely prescribed by medical and paramedical personnel in these countries. Preliminary results from a study in Sri Lanka, show about 10% of the patients, being blood filmed for malaria, had chloroquine in their urine. Therefore, patients with possible malaria presenting to a doctor, or another health worker, with possible malaria may already have taken some antimalarials. A quick, easy method to detect such drugs could help in the clinical management; such a method could also be useful in field studies where the presence of antimalarials needs to be determined easily and quickly.

Clinical Needs

At present, the majority of the patients with malaria are treated by a doctor, or a health worker, with limited facilities. The field assays which could be used in such situations would be limited to relatively simple urine test. Examples of these tests are Bromothymol Blue, (BTB), (Bergqvist et al., 1985), the modifications of Haskins, (Mount et al., 1987), and the Saker Solomons, (SSCQ), (Mount et al., 1989); these test are for chloroquine and are positive when the total concentration of the drug and metabolite are above 1 mg/L. Therefore, this section will concentrate on the possible influence of these tests on the treatment of malaria. When simple tests for other antimalarial drugs are developed, their value will have to be evaluated separately as the problems with these drugs may be different to those with chloroquine.

The therapeutic concentration of chloroquine has not yet been satisfactorily defined. Indeed, it may not be possible to define this level due to the influence of factors such as immunity, the duration of the attack, nutritional status, etc. The field tests, which have a "turn around time" short enough to be useful in clinical practice, use urine. Some correlation between urine and blood levels has been established, though it is insufficient to define a urine level that would indicate an adequate blood concentration. Therefore, an absolute level of chloroquine, which must be detected by a urine test for the drug, is not available.

How would the knowledge of the presence, or absence, of chloroquine in urine influence the treatment of malaria? In situations where the malaria is uncomplicated, and satisfactory

response to treatment with chloroquine is to be expected, a urine test would not influence treatment.

Where chloroquine resistance is suspected after "some" treatment for malaria, a urine test for the drug would be useful. All too often the patient has been treated with more than one drug and does not know what these are: additionally the treatment records are not available. If chloroquine is present in the urine in substantial amounts, it would support a decision to use "second line" drugs. However, the results of these tests will not indicate that a full course of chloroquine has been given; an incomplete course can also give substantial amounts of chloroquine in the urine. Therefore, results of such tests will have to be interpreted along with the clinical history, however imperfect that may be. If chloroquine is administered, the urine could then be checked to verify that absorption has occurred.

Sudden death has been reported after parenteral chloroquine in children, (Williams, 1966). This may have been due to parenteral chloroquine producing toxic concentrations when added to the existing drug concentrations which were the result of recent oral administration. A simple urine test to detect chloroquine, before parenteral administration of the drug, could warn of the potential danger.

Drug interactions involving chloroquine could be avoided with a urine test that detects the drug. Chloroquine and quinine have well known cardiac depressant effects which are dose related; mefloquine too may have cardiac effects. These combined effects could have an additive, or synergistic, effect on the myocardium. If chloroquine can be identified in substantial amounts in the urine, appropriate care could be taken when administering the second drug.

The test described above could also be used after treatment to assess whether chloroquine has been absorbed. Vomiting after chloroquine ingestion is a well known side effect which creates doubt about the absorption of the drug. A rigor immediately before, or after, treatment might also decrease absorption, (Weerasuriya et al., 1989). If chloroquine is detected in the urine a few hours after administration, it is unlikely that poor absorption of the drug is a reason for treatment failure. A semi-quantitative test, such as Saker Solomon CQ II, (Mount et al., 1989), might even provide a rough measure of the degree of absorption.

False Positives and Negatives

The simple urine tests for chloroquine which have been reported, produce false results to varying degrees. What would be the effect of a false positive? A second line drug could be used instead of chloroquine and should result in cure. However,

a large number of false positives would lead to under use of chloroquine, and unnecessary use of more expensive second line drugs. A false negative may lead to a dose that could produce toxic level in combination with that already administered.

In summary, a person treating malaria would ask of these tests. "If this test is negative, is it safe to administer parenteral chloroquine?" Or vice versa.

A qualitative result, with the limit set at the appropriate level, or, at the most, a semiquantitative result, would provide the answer. High specificity would be required but no extreme sensitivity. Further work, especially on blood and urine concentrations seen after a full course of chloroquine needs to be done. Such work would help to decide whether a full course of chloroquine has been given when chloroquine is detected in the urine.

Laboratory, rather than field, tests are required to detect antimalarials in the blood. Recently, ELISA tests which could be used in the smaller hospitals have been described, (Rowell et al., 1988). Further evaluation is required to assess its applicability in the hospitals in areas where malaria is prevalent.

Research Needs

Chloroquine use, whether taken as instructed, or taken without instructions, its often assessed in research studies.

In trials on chemoprophylaxis of malaria with chloroquine, it is important to verify whether the subjects have been taking the drug. Failure in prophylaxis could be due to resistance of the malaria parasite to chloroquine, or to non-compliance; the incidence of the former shows a steady increase, and the latter is commonly due to the side effects produced by the drug. The responses required are obviously different. In the field, a test, which would detect chloroquine up to the end of the dosing interval, would help in differentiating these two important causes of failure of prophylaxis. Malaria with a positive urine test for chloroquine might indicate resistance, whereas a negative test would suggest non compliance.

In vitro testing for drug resistance is a part of the system that monitors the effectiveness of the treatment of malaria; it is also an important part of many research studies. Samples from patients already taking antimalarials need to be excluded from in vitro tests as they could produce misleading results. A test for chloroquine in urine would help in rapidly assessing whether a patient has taken the drug.

In chemoprophylaxis trials, and when taking samples for the vitro testing, it is important to have a test that is

accurate, and provides a quick results, as the next step would depend on the result of the test. However, the absolute level of chloroquine may not be important.

The drug pressure within a community could contribute to the emergence of resistance. Community surveys of chloroquine in the urine would provide a good indication of the drug use. The sensitivity of the test would have an important influence on the result; the more sensitive the test, the greater is the possibility of detecting chloroquine use, as the drug has a long elimination half life. Therefore, it is necessary to define before the study, the period of use being assessed. As the currently available field tests can only detect levels above mg/L, the period of use being assessed would be in weeks rather than months. Some compromise in the specificity could be made for the sake of ease of use in the field, provided sensitivity is unaffected. The false positives could be weeded out in a central laboratory where a more specific test requiring sophisticated equipment would be available. The ELISA for chloroquine might be suitable as the test in the laboratory, as it is more specific than SSCQ and similar tests. ELISA would also provide a quantitative analysis. However, its greater sensitivity would not be exploited as the screening tests would not detect concentrations below 1 mg/L.

A good screening test for chloroquine in urine in the field would also allow more selective blood sampling for chloroquine levels. The chloroquine concentration in the blood, along with the clinical history, might provide a good indication of when the tablets were taken.

Chloroquine, and its metabolite, are concentrated to a great degree in the kidney and are excreted in the urine. Therefore, both are present in a markedly higher concentration in urine when compared to blood. Hence, if the aim is to detect the presence of the drug in the body, urine, rather than blood, should be assayed. Collection of the sample would be easier too. Blood sampling would necessary in some situations, such as when the aim is to define therapeutic concentration of the drug in blood.

In summary, the questions posed by research studies are more complex than the ones in a clinical setting. Therefore, a wider variety of tests is necessary. A test which is excellent in one aspect, (e.g. specificity), but equivocal in another might be ideal for a particular study, but unsuitable for another. As in tests for a clinical setting, further work needs to be done.

Cost

Malaria is basically a disease of developing countries. In some, it is the biggest health problem. Therefore, any

procedure/treatment has to be cheap if it is to be widely used. The cost of a course of chloroquine has been estimated to be 6 US cents, and that of a blood film for diagnosis to be 30 US cents. Hence, the cost of a urine test for chloroquine should be comparable. A test which utilizes equipment in common use, (test tubes, pipettes), would be preferable to one that needs more sophisticated equipment. The Benedict's Solution test for "sugar" in urine would be a good example of simplicity and low cost.

References

Bergqvist, Y., Hed, C., Funding, L. & Suther, A. (1985) Determination of chloroquine and its metabolites in urine: a field method based on ion-pair extraction. Bulletin of the World Health Organization, 63, 893-898.

Mount, D. L., Patchen L. C., Williams, S. B. & Churchill, F. C. (1987) Colorimetric and thin-layer chromatographic methods for field assay of chloroquine and its metabolite in urine. Bulletin of the World Health Organization, 65, 615-623.

Mount, D. L., Nahlen, B. L., Patchen, L. C. & Churchill, F. C., (1989) Adaptations of the Saker-Solomons test: simple reliable colorimetric field assays for chloroquine and its metabolite in urine. Bulletin of the World Health Organization, 67, 295-300.

Rowell, V., Rowell, F. J., Baker, A., Laurie, D. & Sidki, A. M. (1988) A specific ELISA method for determining chloroquine in urine or dried blood spots. Bulletin of the World Health Organization, 66, 211-217.

Weerasuriya, K., Mahindaraine, M. P. D., Pereira, P. L. D. S, Wijeratne, D. T. U. & Tillekeratne, L. M. V. (1989) Plasma concentrations of chloroquine and its metabolite after oral administration in uncomplicated malaria. European Journal of Clinical Pharmacology, 36 (Supplement), A122.

Williams, A. R. F. (1966) Malaria in children. British Medical Journal 2, 1531.

CONCLUSIONS ON THE CURRENT STATUS OF THE DEVELOPMENT OF DETECTION METHODS FOR ANTIMALARIAL DRUGS IN BODY FLUIDS

The results of the comparison of the field laboratory methods for the detection of antimalarials in urine as evaluated in the Penang II Workshop are detailed in Annex 8. On the basis of these results it has been possible to prepare a table (see Annex 9) in which the potential application of each of the currently available test systems is detailed in terms of:

- which body fluids can be tested
- actual, or estimated, sensitivity of the test in mg or ug per litre of test fluid
- minimum training level of the personnel who could be expected to reliably carry out the test procedure and the additional training required by this level of staff to achieve this reliability
- capital material required for the test procedure
- cost of consumables
- cost per test (exclusive of manpower and infrastructure costs)
- level(s) at which test is likely to find application and purposes for which it may be used
- degree of portability: volume and weight
- requirements for safe disposal of used material and waste products resulting from the test procedure

It is to be noted that, for reasons of conformity and clarity, throughout this document all drug concentration levels have been given in SI units - i.e. milligramme per litre (mg/L), microgramme per litre (ug/L) - and where other bastard units have been quoted from reference documentation these too have been converted to the equivalent SI unit.

RESULTS OF A COMPARISON OF FIELD LABORATORY METHODS FOR THE
DETECTION OF ANTIMALARIALS IN URINE
(WHO/TDR WORKSHOP II - PENANG 1989)

Test	Qualitative Visual	Quantitative						Other Biological Fluids
		Chloroquine	Sample Size	Mefloquine	Sample Size	Quinine	Sample Size	
S-S CQ1 (Format A)	Yes	1 mg/L	2 ml	2 mg/L*	2 ml	1 mg/L	2 ml	None
S-S CQ1 (Format B)	Yes	1 mg/L	2 ml	2 mg/L*	2 ml	1 mg/L	2 ml	None
Haskins MM II	Yes	1 mg/L	2 ml	< 2 mg/L**	2 ml	1 mg/L	2 ml	None
TLC (HPTLC plates)	Yes (CQ, DCQ)	1 mg/L	25 ul	1 mg/L Scanner	100 ul	1 mg/L	25 ul	Field: Urine Lab.: Plasma, blood, urine
Elisa (Rowell's Modification)	Yes	0.02 mg/L (urine)	5 ul or <					Blood
Elisa (Eggelte's Modification)	Yes	0.02 mg/L (urine)	5 ul or <	0.03 mg/L (urine)	5 ul or <	0.05 mg/L (urine)	5 ul or <	Blood

LEGEND

* Not detectable in treated patients.

** Not likely to be detectable at the drug concentration levels achieved in treated patients.

Annex 9

DETECTION OF ANTIMALARIAL DRUGS IN BODY FLUIDS: CURRENT STATUS OF DEVELOPMENT
WHO/TDR WORKSHOP II - PENANG 1989
(SEE LEGEND FOR ABBREVIATIONS GIVEN IN PARENTHESES)

TEST SYSTEM AND TEST	TEST FOR WHICH BODY FLUID(S)	TEST DRUG(S) ESTIMATED AND SENSITIVITY	SKILL RATING ADDITIONAL TRAINING TIME	CAPITAL MATERIAL REQUIRED	COST OF CONSUMABLES	COST PER TEST	CURRENT STATUS OF DEVELOPMENT	LIKELY APPLICATION	PORTABILITY	SAFE DISPOSAL ARRANGEMENTS
CHEMICAL										
S-S CQ 1 (Format A)	Urine	CQ QN 1mg/litre PRO	(Mcrst + 2 days)	Pipetter	\$50.00	\$0.20	Ready	(A: 2,3; B: 1,2,3; C: 1,2,; D:2)	Less than 10 kg and 0.1m3	Minimal problems
S-S CQ 1 (Format B)	Urine	CQ QN 1mg/litre PRO	(Mcrst + 2 days)	Pipetter	\$200.00 for 1000 tests	\$0.20	Kit form ready for development	(A: 2,3; B: 1,2,3; C: 1,2)	Less than 10kg and 0.1m3	Care and disposal glass ampoules
Haskins MM II	Urine	CQ QN 1mg/litre PRO	(Mcrst + 4 days)	Filter, photo- meter	\$500.00	\$0.05	Ready	(A: 2,3; B: 1,2,3; C: 1,2; D: 1,2)	Less than 10kg and 0.1m3	Special arrangements disposal of chloroform

LEGEND

Format A = Uses screwcap conical tubes.

Format B = Uses precharged vacuum ampoules.

System A = Small scale assays

System B = Large scale assays

Mcrst + = Microscopist level of training plus number of additional days of in-service training required to achieve proficiency in test procedure.

Technologist + = Technologist level of training plus number of additional days of in-service training required to achieve proficiency in test procedure.

A = Premedication: 1 = field level PHC; 2 = in vivo/in vitro; 3 = treatment management.

B = Post-medication studies: 1 = Compliance; 2 = Absorption; 3 = Side effects.

C = Distribution of drug: 1 = Use by population; 2 = level in population.

D = Research applications: 1 = Laboratory level; 2 = Field level.

TEST SYSTEM AND TEST	TEST FOR WHICH BODY FLUID(S)	TEST DRUG(S) ESTIMATED AND SENSITIVITY	SKILL RATING ADDITIONAL TRAINING TIME	CAPITAL MATERIAL REQUIRED	COST OF CONSUMABLES	COST PER TEST	CURRENT STATUS OF DEVELOPMENT	LIKELY APPLICATION	PORTABILITY	SAFE DISPOSAL ARRANGEMENTS
<u>THIN LAYER CHROMATOGRAPHY</u>										
TLC	Urine	CQ/DCQ (System A) 1mg/litre	(Mcst + 1 day)	Graduated pipetter, UV lamp	\$200.00 for 1000 tests	\$0.30	Ready			
	Urine	CQ/CCQ (System B) 1mg/litre	(Mcst + 1 day)	As above + linear chamber	\$650.00	\$0.20	Kit form to be developed	(A: 2,3; B: 1,2,3; C: 1,2; D: 1,2)	3 kg and 0.1m3	No. Can be reused
	Urine	QN 1mg/litre	(Mcst + 1 day)	As above	\$650.00	\$0.20	Ready			
	Urine	AMO (To be developed)	(Mcst + 1 day)	As above	\$650.00	\$0.20	Further development			
	Urine	MF? CP? SDX? PYR	(Mcst + 1 day)	As above	\$650.00	\$0.20	Further development			

(SEE LEGEND FOR ABBREVIATIONS GIVEN IN APPENDIX 2)

DETECTION OF VITAMINIZING DRUGS IN BODY FLUIDS: CURRENT STATUS OF DEVELOPMENT

Not detectable in treated patients.

VALUE 0

Not likely to be detectable at the drug concentrations listed and/or in treated patients.

TEST SYSTEM AND TEST	TEST FOR WHICH BODY FLUID(S)	TEST DRUG(S) ESTIMATED AND SENSITIVITY	SKILL RATING ADDITIONAL TRAINING TIME	CAPITAL MATERIAL REQUIRED	COST OF CONSUMABLES	COST PER TEST	CURRENT STATUS OF DEVELOPMENT	LIKELY APPLICATION	PORTABILITY	SAFE DISPOSAL ARRANGEMENTS
HPTLC	Whole blood, plasma, serum and urine	CQ/DCQ 5ug/litre	(Technologist + 30 days)		\$23000.00	\$0.15	Ready			
	Whole blood, plasma, serum and urine	QN 5ug/litre	(Technologist + 30 days)	Applica- tion system, linear	\$23000.00	\$0.15	Ready	(A: 2,3; B: 1,2,3; C: 1,2;	Not possible	No problem in lab. conditions
	All body fluids	AMO 10ug/litre	(Technologist + 30 days)	chamber, scanner	\$23000.00	\$0.15	Ready			
	All body fluids	MF 5ug/litre	(Technologist + 30 days)		\$23000.00	\$0.15	Further development			
	All body fluids	CP? SDX? PYR?	(Technologist + 30 days)		\$23000.00	\$0.15	Further development			

LIST OF PARTICIPANTS

TEST SYSTEM AND TEST	TEST FOR WHICH BODY FLUID(S)	TEST DRUG(S) ESTIMATED AND SENSITIVITY	SKILL RATING ADDITIONAL TRAINING TIME	CAPITAL MATERIAL REQUIRED	COST OF CONSUMABLES	COST PER TEST	CURRENT STATUS OF DEVELOPMENT	LIKELY APPLICATION	PORTABILITY	SAFE DISPOSAL ARRANGEMENTS
ELISA										
Rowell's modification	Blood, urine	CQ 25ug/litre PQ 25ug/litre QN 25ug/litre	(Technologist + 10 days)	Reagents, micro- pipetter, plate reader, incubator	\$100.00 to \$150.00	\$1.00 to \$1.50	Ready to within 6 months	Research studies	2 to 3kg 0.2 to 0.3 m3 + plate reader	No problems
Eggelte's modification	Blood, urine	CQ 25ug/litre MF 25ug/litre QN 25ug/litre	(Technologist + 10 days)	Reagents, micro- pipetter, plate reader, incubator	\$3250.00 (1000 tests)	\$0.30 to \$0.75	Ready to within 6 months	Research studies	2 to 3kg 0.2 to 0.3 m3 + plate reader	No problems

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